Aggregate Formation of rCHO Cells and Its Maintenance in Repeated Batch Culture in the Absence of Cell Adhesion Materials

SHINJIRO YAMAMOTO, HIROKI MATSUDA, YASUNORI TANJI, and HAJIME UNNO

Department of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501 and Department of Chemical System Engineering, Yokohama National University, 79–5 Tokiwadai, Hodogaya-ku, Yokohama 240–8501, Japan

Received 1 November 1999/Accepted 28 February 2000

Aggregate formation of recombinant Chinese hamster ovary (rCHO) cells capable of producing granulocyte colony-stimulating factor (G-CSF), using medium lacking cell adhesion materials in a repeated batch culture, was examined together with cell growth, cell viability and G-CSF production. The rCHO culture was conducted in a rotary shaker and the medium was changed every five days. The formation of stable cell aggregates with high reproducibility was observed after the first medium change. The size of the cell aggregates (consisting of several 10s to 40,000 cells) formed during the repeated batch culture ranged from 30 to 600 μm. The cell density of the aggregates reached as high as 2 × 10^6 cells/ml and the viability was maintained at more than 80% for 19 d. Changing the medium to avoid glucose exhaustion effectively maintained the cell density, cell viability and G-CSF productivity at high levels.

[Key words: cell aggregate, repeated batch culture, rCHO, G-CSF]

Animal cell culture has been used to produce many kinds of therapeutic and diagnostic proteins in pharmaceutical manufacturing. Most animal cells exhibit anchorage-dependent growth requiring cell attachment to the surface of solid materials. Microcarrier culture is a useful method to obtain a high cell density of anchorage-dependent animal cells, and facilitates the separation of cells from the medium for repeated cell usage. Since cell culture methods using any cell adhesion materials are expensive to perform, aggregate cell culture without using such materials is considered to be an alternative approach to the economical production of bioactive compounds (1–7). Anchorage-dependent animal cells, such as baby hamster kidney (BHK) (8–10), Chinese hamster ovary (CHO) (11), Vero (12) and human kidney 293 (1) have been shown to form cell aggregates resembling tissues in a stirred tank bioreactor in the absence of microcarriers. Cells inside the aggregates were protected from fluid shear stress in the reactor (1), which makes it possible to maintain both cell viability and the productivity of target biochemicals in large-scale culture where high agitation speed is used to avoid limiting the oxygen supply. Moreover, it has been reported that cell aggregation promotes cell-cell interaction and cell function, resulting in the enhancement of metabolite production (3, 13, 14; Shiragami, N. et al., Proc. the 59th Annu. Meet. Soc. Chem. Eng., Japan, E103, 1994 (in Japanese)).

Repeated use of stable cell aggregates is one strategy for the economical production of cell metabolites. In this study, conditions under which stable animal cell aggregates were obtained without using cell adhesion materials, and culture methods which make it possible to utilize the aggregates repeatedly were investigated using rCHO cells, with respect to cell density, cell viability and G-CSF productivity.

**MATERIALS AND METHODS**

**Cells and culture medium** Recombinant chinese hamster ovary (rCHO) cells capable of producing granulocyte colony-stimulating factor (G-CSF) were used. The culture medium was a mixture of Dulbecco’s Modified Eagle’s medium (DMEM) and F-12 medium (Dainippon Pharmaceuticals, Tokyo) at a ratio of 1:1, supplemented with 10% foetal calf serum (Cansera International Inc., Canada). Antibiotics used were penicillin G (100/11/ml) and streptomycin (100 μg/ml) (Gibco BRL Life Technol. Inc., USA).

**Culture** Subculture of the cells was conducted in a 1-flask under a humidified 5% CO₂ atmosphere at 37°C. Cells (2 × 10^2 cells/ml) harvested from the subculture using a 0.25% trypsin-EDTA solution for 5 min were inoculated into the wells of a 6-well plate, each well containing 2 ml of medium, which was incubated under a humidified 5% CO₂ atmosphere at 37°C. The inner well surface was chemically modified using poly-2-hydroxyethyl-methacrylate (Sigma, USA), a cell attachment inhibitor (15). Cells were cultured at 60 rpm in a rotary shaker (MMS, Eyela, Tokyo) and the medium was changed every five days.

**Cell density and cell viability** Total cell densities and the number of viable cells were measured by the methods of cell counting and trypan blue exclusion, respectively. Cell viability was obtained by dividing the viable cells density by the total cell density. Cell aggregates were dissociated in a 0.25% trypsin-EDTA solution for 20 min which did not damage the cells.

**Analysis** Glucose concentration was measured using a glucose kit (GLU NEO, SHINO-TEST Corp., Tokyo).

G-CSF was separated by reverse phase HPLC using a silica column (YMC-Pack C8-AP; 250 × 4.6 mm, YMC Co., Tokyo). The column was equilibrated with 30% propanol in 0.1% trifluoroacetic acid (TFA) for 16 min. G-CSF was eluted at a flow rate of 0.6 ml/min under a
linear gradient of propanol from 30% to 52.5% in 0.1% TFA over 22.5 min and detected by their absorbance at 220 nm.

G-CSF productivity was calculated using Eq. 1.

\[
\text{G-CSF productivity} = \frac{[\text{G-CSF}]_{t_2} - [\text{G-CSF}]_{t_1}}{(X_{t_2} + X_{t_1}) (t_2 - t_1)}
\]

where \([\text{G-CSF}]_t\) and \([X]_t\) are the G-CSF concentration and cell density at time \(t\), respectively.

Size of cell aggregates

The diameter of cell aggregates was determined using an image analyzer (Luzex-F, Nireco, Tokyo). Based on the assumption that the aggregate is a rotational ellipsoid, the long and short axes being \(a\) and \(b\), respectively, the volumetric equivalent diameter of the aggregate, \(R\), was calculated using Eq. 2.

\[
R = \sqrt[3]{\frac{a b^2}{2}}
\]

Observation of cell aggregates

Cell aggregates were observed using a scanning electron microscope (Superscan 330, Philips, Netherlands) and a modification of a method reported previously (16).

RESULTS AND DISCUSSION

Formation of cell aggregates in repeated batch culture

Aggregate formation of CHO and BHK cells, which are widely used in industry for the production of useful biochemicals in the absence of cell adhesion materials, has been reported for cells grown in suspension culture in stirred vessels (1, 2, 8, 11, 12). Although Goetghbeur and Hu reported on the cell aggregate formation of CHO cells in a stirred bioreactor, rCHO cells used in this study did not always form such aggregates under conditions similar to those used by Goetghbeur and Hu (11). The formation of cell aggregates is considered to be related to both cell surface characteristics which promote cell attachment to the culture vessel and to cell-cell contacting conditions in a bioreactor. To achieve the reproducible formation of cell aggregates of rCHO cells, culture of rCHO cells was carried out in a vessel mounted on a rotary shaker, where most of the cells were captured in the vortex flow of the medium (17) and the medium was changed repeatedly.

In the initial culture stage under conditions of low cell density of less than 1x10^6 cells/ml no aggregates were observed, while stable cell aggregates appeared two days after the first medium change, when the cell density increased to approximately 2x10^6 cells/ml and the cell viability was more than 95%. These stable aggregates, which were not dissociated by pipetting, were formed with high reproducibility, and grew in subsequent cultures. On the contrary, when a larger inoculum size (2x10^6 cells/ml) was used, the cells aggregated in the early culture stage. However, the aggregates formed were very fragile and easily dissociated by pipetting.

Renner reported that deoxyribonucleic acid (DNA) released from decaying and dead cells mediated cell-cell adhesion and aggregation (18). That we observed a dead cell density of five percent (cell viability of ninety five percent) following aggregate formation in the present study supports such a mechanism. However, the stable aggregates of rCHO cells could not be dissociated by treatment with DNase (data not shown). The aggregates were dissociated following incubation in a 0.25% trypsin-EDTA solution for 20 min while dissociation was not observed following incubation in a trypsin-EDTA solution for 5 min. These results suggest that extracellular matrix (ECM) proteins such as fibronectin and glycoproteins, accumulated during long culture periods. Such long periods might be necessary for stable aggregate formation because several days are needed for ECM protein production (19) after trypsin treatment.

Size distribution of cell aggregates

Figure 1 shows the distribution of cell aggregate size as a function of culture time in the repeated batch culture. The size of the cell aggregates (consisting of several 10s to 40,000 cells) ranged from 30 to 600 μm. The cell aggregates of size 600 μm were maintained in the subsequent cultures. The distribution of cell aggregate size shifted to larger cell aggregates with culture time, which reflected the increase in the mean diameter of the cell aggregates. The number of cell aggregates formed after 15, 25 and 30 d of culture were 411, 370 and 323 ml^-1, respectively. The decrease in the number of cell aggregates, at an almost constant cell density, after 15 d of culture (shown in Fig. 3a) suggested that cell aggregates of less than 600 μm were formed as a result of aggregate-aggregate and/or aggregate-suspended single cell attachment, together with the proliferation of individual cells constituting the aggregate during the culture.
FIG. 2. Relationship between cell density and cell viability in cell aggregates formed from 12 to 31 d of culture during repeated batch culture.

The dependency of the density and viability of cells in the aggregates on aggregate size during the repeated batch culture is shown in Fig. 2. The data were obtained from arbitrarily selected aggregates formed from 12 to 31 d of culture. A cell density of more than $3 \times 10^6$ cells/ml-aggregate and an 80% cell viability in aggregates ranging from 100 to 600 μm in size were obtained. Cell density in aggregates had a tendency to decrease with increasing aggregate size. From SEM observations, cells in the aggregates of the smaller size were more densely packed, which reflects the increase of cell density in smaller aggregates.

The formation of large aggregates of cells of various viable cell lines has been reported: BHK cell aggregates of 200 μm (20), Vero cell aggregates of 250 μm (11), CHO cell aggregates of up to 360 μm and Swine Testicular cell aggregates of up to 380 μm (11); the viability of the cells of these aggregates was routinely higher than 85%. The viability of rCHO cells forming aggregates of less than 200 μm in this study was greater than 85%, which is similar to the above results. More than 80% cell viability of rCHO cells in aggregates up to 600 μm suggests that the cells inside the aggregates were quiescent, and that sufficient quantities of nutrients and oxygen were supplied to the inner cells by way of gap junctions which facilitate nutrient transfer from cell to cell (21).

Cell growth, glucose consumption and G-CSF production in repeated batch culture

Figure 3a shows the time course of cell density and viability in repeated batch culture. Cell aggregates were formed after 7 d culture and maintained in subsequent cultures. A cell density of $2 \times 10^6$ cells/ml and viability of more than 80% were maintained after the formation of the cell aggregates up to 31 d, then the aggregate size and the cell viability tended to decrease rapidly. This decrease was due to glucose exhaustion as shown in Fig. 3b. The glucose consumption rate after aggregate formation was relatively low compared with that of the cells before the first medium change, which suggested the existence of a quiescent region inside aggregates.

In our previous study a maximum cell density of $1.5 \times 10^6$ cell/ml was maintained by changing the medium every day (14). In this study, a cell density of more than $2 \times 10^6$ cells/ml was maintained from 12 up to 31 d after inoculation by changing the medium every 5 d. These results indicate that it is possible to conduct an aggregate culture of high cell density in the absence of cell adhesion materials, which reduces medium cost.

The accumulation of G-CSF in medium was monotonous after the formation of cell aggregates as shown in Fig. 3c. A similar pattern of G-CSF productivity before and after the formation of aggregates was observed as shown in Fig. 3d, indicating that G-CSF synthesis by rCHO cells was hardly affected by the formation of cell aggregates. The significant increase in G-CSF productivity after changing the medium was due to the fresh supply of glucose.

Operational procedure for increasing the cell growth

FIG. 3. Cell density and cell viability (a), glucose concentration (b), G-CSF concentration (c) and G-CSF productivity (d) in repeated batch culture. Broken lines indicate assumed pattern of G-CSF productivity in (d). Bars represent scattered data range of three samples.
and G-CSF production To maintain G-CSF production at a high level in the aggregate culture for long periods, a limitation in the supply of glucose has to be avoided.

Before glucose exhaustion occurred after 12 d of culture, the effects of glucose supply, addition of glucose only (B), changing the medium (C) and 12 d of culture without a medium change (A), as shown in Fig. 4, were examined. As shown in Figs. 5a and 5b, cell density, cell viability and G-CSF production in both (B) and (C) cultures, where glucose was not a limiting nutrient, were increased compared with culture (A) after 13 d of culture. Cell density and cell viability in culture (C) were maintained during the culture while those in culture (B) decreased after 14 d of culture. The decrease was assumed to be due to the accumulation of growth inhibitors such as ammonium ions and lactate. These results indicate that glucose addition to the medium is necessary to maintain cell density, cell viability and G-CSF production at high level during aggregate culture, and changing the medium, which removes growth inhibitors, is preferred for the subsequent long term culture.

In the present cell aggregate culture, the formation of stable cell aggregates, and the maintenance of a high cell density of 2 × 10⁶ cells/ml and cell viability of more than 80% for the cells in aggregates of less than 600 μm for 19 d in the repeated batch culture suggest that the cell aggregates are suitable for long term culture. Since cell aggregate size is one of the factors affecting the cell growth because of diffusion limitation of nutrients and oxygen, the effect of controlling aggregate size on cell viability and metabolite production should be investigated in future studies.

Conclusion Highly reproducible stable cell aggregates were formed after the first medium change and maintained in repeated batch culture where the medium was changed every five days without using cell adhesion materials. A high cell density of 2 × 10⁶ cells/ml, a cell viability of more than 80% and a high level of G-CSF production were maintained for cell aggregates of less than 600 μm for 19 d of culture.

Methods of supplying glucose, which was a limiting nutrient, were examined to obtain cell aggregates maintaining a high cell density, cell viability and G-CSF production, and among these methods changing the medium was found to be most effective.

ACKNOWLEDGMENTS

We thank Chugai Pharmaceutical Co., Tokyo for supplying rCHO cells.

REFERENCES


10. Moreira, J. L., Alves, P. M., Aunins, J. G., and Carrondo, M. J. T.: Hydrodynamic effects on BHK cells grown as suspend-