Comparison of different hepatocyte cell lines for use in a hybrid artificial liver model

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Abstract

Selection of a cell line suitable for a hybrid artificial liver model employing cellulose porous beads (CPBs) was investigated. Hep G2 cells grown in a culture dish exhibited appreciably higher ureogenesis and gluconeogenesis activities than those grown in CPBs. SEM observation of CPBs revealed marked difference in the distribution of attached cells from one bead to another, and showed that almost all the cell-bearing micropores were completely packed with cells.

With the aim of selecting a cell line not prone to excessive aggregation and which grows moderately so as not to fill up the micropores, cells of 6 cell lines, HLE, HLF, Hep 3B, PLC/PRF/5, Huh 7 and Hep G2, were cultivated in dishes. Hep G2, HLE, and HLF increased to 5×10^5 cells/cm², whereas PLC/PRF/5 grew only to 5×10^4 , and Hep 3B and Huh 7 up to 2×10^4 cells/cm². The specific activities of ureogenesis and gluconeogenesis of Huh 7 were the highest among the lines tested – 42- and 7-fold those of Hep G2, respectively. When the 6 cell lines were grown in a submerged culture with 0.6 g/l of CPBs, Huh 7 had the lowest cell concentration of 0.54×10^6 cells/ml, and the highest activities of ammonia consumption and urea and glucose production (1.38 μ mol NH₃, 99 nmol urea, and 14.5 nmol glucose/10⁶ cells/h). Consequently, Huh 7 is considered to be a suitable cell line for use in the development of an artificial liver model employing porous beads.

Introduction

Generally, a small internal volume is considered to be essential for artificial organs such as an artificial liver because the volume of blood circulating through the organ is likely to be small. Because of this requirement, several types of hybrid artificial liver models which aim to achieve a high cell density and a high level of activity have been investigated. While the cell density in a hollow fiber module bearing hepatocyte cells in the extracapillary space (Sussman *et al.*, 1993, Takeshita *et al.*, 1993), is rather lower than that of liver tissue (1×10^8 cells ml⁻¹), hollow fiber modules with collagen gel in the extracapillary (Sakai *et al.*, 1994) or intracapillary space (Nyberg *et al.*, 1993), as well as those with microcarriers in the extracapillary space (Rozga *et al.*, 1993), can contain 6–10 $\times 10^6$ cells ml⁻¹. However, these reactors are considered to be inferior due to the low mass transfer rate between the cells and the

blood because of diffusion limitation. Compared with these membrane reactors, a packed-bed reactor with a porous carrier is attractive from the view point of both cell density and mass transfer, because soluble components in the blood are transferred not by diffusion but by convection between the cells and the blood (Park *et al.*, 1993). The reason why the cell densities in packed-bed reactors employing polyurethane foam (Ijima *et al.*, 1993) and polyvinyl foam resin (Miyoshi *et al.*, 1993) were only about $1\sim5 \times 10^6$ cells ml⁻¹ bed may be the large pore diameter of 200–250 μ m and a low affinity between the cells and the bead material. An alternative porous material with a small pore diameter and high affinity is the cellulose porous bead (CPB) manufactured by Asahi Chemical Industry Co. Ltd., which has a pore diameter of less than 100 μ m, and whose surface can be modified with DEAE group ions to have a positive charge (Takagi *et al.*, 1994).

Almost all the artificial liver models referred to above utilized porcine or rat hepatocyte cells, because human and animal hepatocyte cells cannot grow in a culture while retaining their differentiated properties. Although there is a virtually inexhaustible supply of porcine hepatocytes, reproducible isolation of such cells on a large scale is particularly difficult. Furthermore, potential incompatibility problems exist regarding animal cell antigens in humans. An alternative approach involves the use of continuous cell lines that retain key differentiated properties of normal hepatocyte cells in culture and can be grown to form a large, homogeneous population. Among the cell lines established from hepatocellular carcinoma, Hep G2 (Aden *et al.*, 1979), HLE, HLF (Doi *et al.*, 1975), Hep 3B, PLC/PRF/5 (Macnab *et al.*, 1976) and Huh 7 (Nakabayashi *et al.*, 1982) have been reported to maintain certain biochemical functions of hepatocytes to a high degree and are available from public cell banks, eg. ATCC, JCRB.

Besides several important clinical criteria such as blood levels of ammonia, urea, lactate, glucose, bilirubin, fibrinogen and prothrombin in particular, avoiding high levels of ammonia and lactate and a low level of glucose are critically important in the treatment of acute liver failure. Consequently, cells in a hybrid artificial organ should maintain high activities of ureogenesis to convert ammonia to urea and gluconeogenesis to produce glucose from lactate.

Consequently, as a starting point for the construction of a hybrid artificial liver model, we investigated the selection of a highly functional cell line which will grow well inside the micropores of cellulose porous beads as well as exhibiting high ureogenesis and gluconeogenesis activities.

Materials and methods

Cell and growth medium

The hepatocyte cell lines Hep G2 (ATCC HB8065), HLE (JCRB404), HLF (JCRB405), Hep 3B (ATCC HB8064), PLC/PRF/5 (JCRB406), and Huh 7 (JCRB403) were employed. DMEM (Gibco Co. Ltd., NY, USA) supplemented with 10% FCS (Gibco), 5,000 U l⁻¹ penicillin and 5 mg l⁻¹ streptomycin (Sigma, MO., USA) was used for cell growth. It was confirmed that the cell lines of Hep G2 and Huh 7 were not infected by mycoplasmas (Aden *et al.*, 1979, Nakabayashi *et al.*, 1982).

Microcarrier

As a porous microcarrier, cellulose porous beads (CPBs) manufactured by Asahi Chemical Industry Co. Ltd. (Tokyo, Japan) were used. The bead and pore diameters were approximately 500 and 100 μ m, respectively, and the surface of the bead material was positively charged with DEAE group ions.

Cell growth cultures

Tissue culture dishes (55 cm², Corning Co. Ltd., USA) containing 10 ml of the growth medium was inoculated with $0.2\text{--}1.0 \times 10^6$ cells and incubated at 37 °C in a incubator with 5% CO₂. Cells grown in the dishes were harvested by trypsinization with 1 ml of trypsin-EDTA solution containing 0.5 g l⁻¹ of trypsin and 0.2 g l⁻¹ of EDTA (Sigma). Trypsin was inactivated by the addition of 3 ml of the growth medium. Ten milliliters of growth medium in spinner bottles (30 ml; Shibata Hario Co. Ltd., Tokyo) was inoculated with $0.6\text{--}1.8 \times 10^7$ harvested cells together with 18 mg of CPBs. This was incubated at 37 °C in an atmosphere of 5% CO₂. After agitation at 70 rpm for 10 sec at intervals of 15 min, repeated 8 times, the culture was incubated without agitation for 4 h. Then, 20 ml of the growth medium was added and continuous agitation at 70 rpm was started. The culture medium was replaced with fresh medium before glucose was depleted. Each cultivation condition was replicated and the data values in all figures and tables were the means of duplicates.

Assays of ureogenesis and gluconeogenesis activities

For assays of ureogenesis and gluconeogenesis activities, the growth medium supplemented with 5 mM ammonium chloride or Krebs-Henseleit buffer containing 10 mM L-sodium lactate was used as the ammonia and lactate loading solutions. In the case of dish-grown cells, after washing with PBS(–) and adding each respective loading solution, cells were incubated for 3 h at 37 °C in a CO₂ incubator. CPBs bearing cells were washed twice with PBS(–) and then transferred into a microbial grade culture dish (27 cm², Corning). They were incubated in the same manner as dish-grown cells. The ammonia, urea, and glucose concentrations were assayed using taken samples and the reaction rates were calculated.

Analysis. Cell concentrations were determined by the nuclei staining method (Sanford *et al.*, 1950). Glu-

Figure 1. Electron microscope observations of CPBs bearing Hep G2 cells. Hep G2 cells were cultivated in a spinner bottle together with 0.6 g l^{-1} of CPBs. A, distribution of attached cells among the CPBs; B, surface of a cell-bearing CPB; C, cross-section of a cell-bearing CPB. The dotted lines in the photos indicate the scales of 1,000, 50 and $200 \mu\text{m}$, respectively.

cose in the cell growth culture medium was measured by the glucose-oxidase peroxidase method, using an auto-analyzer (Biochemistry Analyzer 2700; YSI Inc., Ohio, USA). Glucose in the loading solution was determined by the mutarotase GOD method (Glucose C II-test Wako, Wako Pure Chemicals Co. Ltd., Osaka, Japan). The ammonia and urea concentrations were determined by the indophenol (Ammonia-test Wako) and diacetyl monoxime (Ureic nitrogen-test Wako) methods, respectively.

Determination of cell aggregation rates

Cells harvested from dishes and suspended at a concentration of $2.5 \times 10^5 \text{ cells ml}^{-1}$ in the growth medium in spinner bottles were incubated with agitation at 70 rpm for 120 min. The total cell concentration and concentration of detached free cells were determined during the incubation by the nuclei staining method and a hemocytometer, respectively. The single cell ratio was calculated by dividing the latter by the former.

Results and discussion

Comparison of the activities of Hep G2 cells grown in CPBs and in dishes

To identify the criteria for the selection of an optimum hepatocyte cell line, the activities of Hep G2 cells, which is reported to be one of the most active hepatocyte cell lines, were compared between cells grown in a dish and those grown in CPBs. Hep G2 cells grew to $1.6\text{--}1.9 \times 10^6 \text{ cells ml}^{-1}$ in both cultures, though the specific growth rate in the CPB culture (0.015 h^{-1}) was lower than that in dish culture (0.037 h^{-1}) (data not shown). Scanning electron microscope (SEM) observation revealed marked differences in the numbers of cell attached to individual CPBs, and the fact that the micropores of some CPBs were fully packed with cells (Figure 1) while those of other CPBs were not. The difference in cell attachment was most likely caused by the rapid aggregation of cells harvested from the dish surface during cell inoculation to the CPB, as mentioned below. The reason for the dense packing of cells in some CPB was considered to be the low contact inhibition of Hep G2 cells.

Dish-grown cells had a specific ammonia consumption rate of $27.7 \text{ nmol}/10^6 \text{ cells h}^{-1}$ and a urea production rate of $7.3 \text{ nmol}/10^6 \text{ cells h}^{-1}$ (Table 1). CPB-grown cells exhibited almost the same specific ammo-

Table 1. Comparison of ureogenesis and glyconeogenesis activities of Hep G2 cells grown in a dish and in a spinner bottle with CPBs

Cell growth	Cell concn. (10^6 cells ml^{-1})	NH_3 consumption rate ($\text{nmol}/10^6$ cells h^{-1})	Urea production rate ($\text{nmol}/10^6$ cells h^{-1})	Glucose production rate ($\text{nmol}/10^6$ cells h^{-1})
Dish	1.63	27.7	7.3	7.0
CPB	1.94	32.0	0.4	-1.1

Figure 2. Comparison of cell aggregation rates. Cells were suspended at a concentration of 2.5×10^5 cells ml^{-1} in the growth medium and incubated in spinner bottles. The time courses of the total cell concentration and single cell concentration were determined, respectively. The ratio of the single cell number to the total cell number was calculated. $\circ$, Hep G2; Δ , HLE; $\square$, HLF; $\bullet$, PLC/PRF/5; $\blacktriangle$, Hep 3B; $\blacksquare$, Huh 7.

nia consumption rate, but their specific urea production rate was only 5% of that of their dish-grown counterparts. Furthermore, while gluconeogenesis activity ($7 \text{ nmol}/10^6$ cells h^{-1}) was present within dish-grown cells, such activity was negative in cells grown in CPBs. The low ureogenesis and gluconeogenesis activities of the CPB-grown cells was considered to be caused by the low mass transfer rate between the interior and exterior of the CPBs. The main products from ammonia were not clear.

Consequently, it is suggested that the criteria for selection of an appropriate cell line for an artificial liver employing porous beads are that the cell should have low cohesiveness in suspension to avoid rapid aggregation, and moderate contact inhibition so as not to fill up to micropores in the CPBs.

Figure 3. Comparison of cell growth in dishes. The 6 cell lines were inoculated into 10 ml of growth medium in culture dishes (55 cm^2). The symbols are the same as those in Figure 2.

Comparison of cell aggregation rates of the cell lines

Cell aggregation rates were determined for the 6 hepatocyte cell lines investigated. Even at 0 min, the ratio of single cells of Hep G2 was only 20%, and it decreased to 0% within 120 min (Figure 2). HLF cells showed almost the same aggregation velocity as those of Hep G2, while HLE aggregated more rapidly. Compared with these three cell lines, the rate of single cells for Hep 3B, PLC/PRF/5 and Huh 7 were higher and well maintained at 40–50%, even after 2h, indicating a high level of single-cell stability. This result showed that Hep 3B, PLC/PRF/5 and Huh 7 were more appropriate cell lines for the development of an artificial liver model because they had low cohesiveness in suspension to avoid rapid aggregation during cell attachment to CPBs.

Figure 4. Microscopic appearances of the 6 hepatocyte cell lines grown in dishes. A, Hep G2; B, HLE; C, HLF; D, PLC/PRF/5; E, Hep 3B; F, Huh 7. These photos were made at the end of the batch.

Comparison of cell densities and activities among the cell lines grown in dishes

Cells of the 6 cell lines were cultured in dishes and their activities were determined after cell growth (Fig-

ure 3). Hep G2, HLF and HLE cells grew to high concentrations of 5.21 , 4.29 and 2.89×10^5 cells cm^{-2} , respectively, whereas the maximum cell concentration of PLC/PRF/5 cells was only 0.28×10^6 cells ml^{-1} and those of Hep 3B and Huh 7 cells were less than

Table 2. Comparison of ureogenesis and glyconeogenesis activities among various hepatocyte cell lines grown in dishes

Cell line	Cell concn. (10^6 cells ml^{-1})	NH_3 consumption rate ($\text{nmol}/10^6$ cells h^{-1})	Urea production rate ($\text{nmol}/10^6$ cells h^{-1})	Glucose production rate ($\text{nmol}/10^6$ cells h^{-1})
Hep G2	2.870	37.5	17.3	8.5
HLE	0.706	7.0	74.2	4.5
HLF	1.200	0.0	16.5	-0.5
PLC/PRF/5	0.281	325.9	24.9	27.4
Hep 3B	0.097	200.0	216.9	13.7
Huh 7	0.060	1850.0	727.2	63.6

Figure 5. Comparison of cell growth in CPBs. The 6 cell lines were cultivated in spinner bottles together with 0.6 g l^{-1} of CPBs. The culture medium was replaced with fresh medium before glucose was depleted and the specific glucose consumption rate was calculated. The symbols are the same as those in Figure 2.

0.1×10^6 cells ml^{-1} (Figure 3). Hep G2 cells were observed microscopically to form a multilayer in dish culture, whereas there were obviously some interstices among the cells in the dish cultures of Hep 3B and Huh 7 (Figure 4). This suggests that Hep 3B and Huh

7 cells possess higher contact inhibition than the other cell lines.

The biochemical activities of cells grown in dishes are summarized in Table 2. The specific rates of ammonia consumption and urea production by Huh 7 cells were apparently the highest among all the cell lines, and were 49 and 42 times those of Hep G2, respectively. The specific glucose production rate by Huh 7 was also the highest of all.

These results suggested that in terms of contact inhibition, ureogenesis and gluconeogenesis activities, Huh 7 is the most appropriate cell line for use in an artificial liver model.

Confirmation of the superiority of specific activities of Huh 7 cells grown in CPBs

The 6 cell lines were cultured with CPBs in spinner bottles (Figure 5). Hep G2 had the highest maximum cell concentration (2.28×10^6 cells ml^{-1}) and Huh 7 the lowest (0.54×10^6 cells ml^{-1}). The final specific glucose consumption rate of Hep G2 was lower than that of any of the other cell lines, which is considered to be caused by mass transfer limitation between the interior and exterior of the pores in the CPBs due to dense packing of cells in CPBs.

The activities of cells grown in CPBs were determined (Table 3). HLF and PLC/PRF/5 cells exhibited ammonia production instead of its consumption, while Hep G2, Hep 3B and PLC/PRF/5 cells consumed urea. Hep G2, HLF and PLC/PRF/5 cells were not net producer of glucose. Consequently, Huh 7 was only the cell line which consumed ammonia and produced urea and glucose like dish-grown cells. Moreover, the rates of these three activities in Huh 7 were amongst the highest. Ammonia consumption rates of 100, 200 and 300 $\text{nmol}/10^6$ cells h^{-1} were reported using the immo-

Table 3. Comparison of ureogenesis and glyconeogenesis activities among various hepatocyte cell lines grown in spinner bottles with CPBs

Cell line	Cell concn. (10^6 cells ml^{-1})	NH_3 consumption rate ($\text{nmol}/10^6$ cells h^{-1})	Urea production rate ($\text{nmol}/10^6$ cells h^{-1})	Glucose production rate ($\text{nmol}/10^6$ cells h^{-1})
Hep G2	2.28	52.8	-20.1	-1.5
HLE	0.78	359.8	-37.9	3.5
HLF	1.20	-393.0	115.6	-0.4
PLC/PRF/5	0.74	-399.7	-1.6	-5.3
Hep 3B	1.15	535.6	-114.7	10.5
Huh 7	0.54	1384.2	99.1	14.5

bilized cells of primary hepatocyte polyurethane foam, hallow fibre containing collagen gel and CPBs, respectively (Ijima *et al.*, 1993, Sakai *et al.*, 1993).

With these results, it was proved that Huh 7 was an appropriate cell line for the development of an artificial liver model.

Conclusion

Among 6 hepatocyte cell lines investigated, only Huh 7 satisfied both criteria of possessing high contact inhibition and high maintenance of single cells in suspension. These characteristics allowed Huh 7 cells to maintain relatively high levels of ureo-genesis and gluconeogenesis activities in both dish and CPB cultures. Consequently, Huh 7 is considered to be the most suitable of the 6 cell lines for use in the development a packed-bed artificial liver employing porous beads.

References

- Aden DP, Fogel A, Plotkin S, Damjanov I and Knowles BB (1979) Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature* 282: 615–616.
- Doi I, Namba M, Sato J (1975) Establishment and some biological characteristics of human hepatoma cell lines. *Gann* 66: 385–392.
- Ijima H, Matsushita T and Funatsu K (1993) Development of a hybrid type artificial liver using PUF/spheroid culture system of adult hepatocytes. *Jpn. J. Artif. Organs* 22: 171–176.
- Kasai S, Sawa M, Tomita I, Hashimoto M, Jiang B, Hirai S, Kakisaka A, Yamamoto T, Mito M and Inoue M (1993) Metabolic activities of multiporous cellulose microcarrier attached hepatocytes. *Jpn. J. Artif. Organs* 22: 159–163.
- Macnab GM, Alexander JJ, Lecatsas G, Bey EM and Urbanowicz JM (1976) Hepatitis B surface antigen produced by a human hepatoma cell line. *Br. J. Cancer* 34: 509–515.
- Miyoshi H, Yanagi H, Furukawa K and Ohshima N (1993) Continuous culture of hepatocytes under different medium conditions using a packed-bed reactor loaded with porous resins. *Jpn. J. Artif. Organs* 22: 147–152.
- Nakabayashi H, Taketa K, Miyano K, Yamane T and Sato J (1982) Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. *Cancer Res.* 42: 3858–3863.
- Nyberg SL, Shatford RA, Peshwa MV, White HG, Cerra FB and Hu W-S (1993) Evaluation of a hepatocyte entrapment hollow fiber reactor: A potential bioartificial liver. *Biotech. Bioeng.* 41: 194–203.
- Park S and Stephanopoulos G (1993) Packed bed bioreactor with porous ceramic beads for animal cell culture. *Biotech. Bioeng.* 41: 25–34.
- Razga J, Holzman MD, Ro M-S, Griffin DW, Neuzil DF, Giorgio T, Mosconi AD and Demetriou AA (1993) Development of a hybrid bioartificial liver. *Annals. Surgery* 217: 502–511.
- Sakai Y and Suzuki M (1994) Functional expressions by hepatocyte spheroids entrapped in collagen gel in hollow fiber modules. *Jpn. J. Artif. Organs* 23: 473–478.
- Sanford KK, Earle WR, Evans VJ, Waltz HK and Shannon JE (1959) The measurement of proliferation in tissue cultures by enumeration of cell nuclei. *J. Nat. Canc. Inst.* 11: 773–795.
- Sussman NL and Kelly JH (1993) Improved liver function following treatment with an extracorporeal liver assist device. *Artif. Organs* 17: 27–30.
- Takagi M, Shirokaze J, Oishi K, Otsubo K, Yamamoto K, Yoshida N and Fujimatsu I (1994) Production of S- (+)-ibuprofen with high optical purity from a nitrile compounds by cells immobilized on cellulose porous beads. *J. Ferment. Bioeng.* 78: 191–193.
- Takeshita K, Ishibashi H, Suzuki M, Tobe S, Takei Y, Kishida A, Akaike T and Kodama M (1993) High cell-density culture system of hepatocytes entrapped in a three dimensional collagen-gel matrix using a hollow fiber module. *Jpn. J. Artif. Organs* 22: 153–158.

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