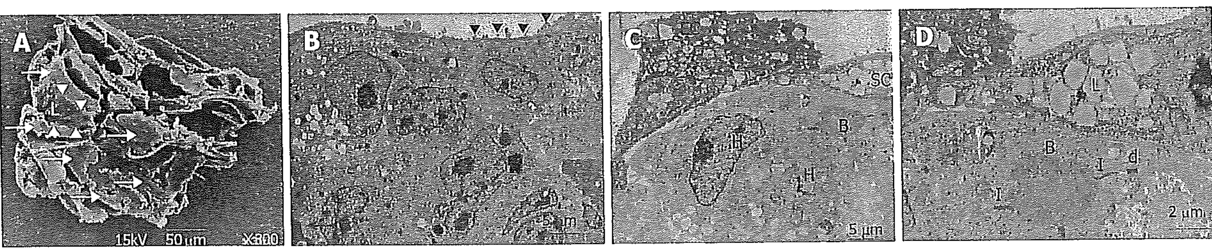


Figure 3 Transmission electron microscopic images of cocultures in the RFB. A: The cells are arrayed on the cellulose beads. Several cell clusters could be seen in a gap of cellulose beads (arrow). Vascular lumen structure surrounding cell clusters could be seen in the beads (small arrow). Culture media flow through inside of lumen structure; B: The cells are arrayed in layers on cellulose beads. Part of a cellulose bead (arrow) is visible at the bottom of the layer. A process of a sinusoidal endothelial cell (arrowhead) is seen at the perfusion side. Scale bar: 5 μm; C: Sinusoidal endothelial cells (EC) can be seen at the perfusion side. Hepatic stellate cells (SC) containing fatty vitamin A droplets are seen overlying the FLC-5 cells (H). FLC-5 cells (H) below EC and SC show bile-canaliculus-like structures (B). Scale bar: 5 μm. D: Bile canaliculus-like structures (B) containing electron dense bile components. Tight junctions (t) and desmosomes (d) are visible, as are fatty vitamin A droplets (L). Scale bar: 2 μm.

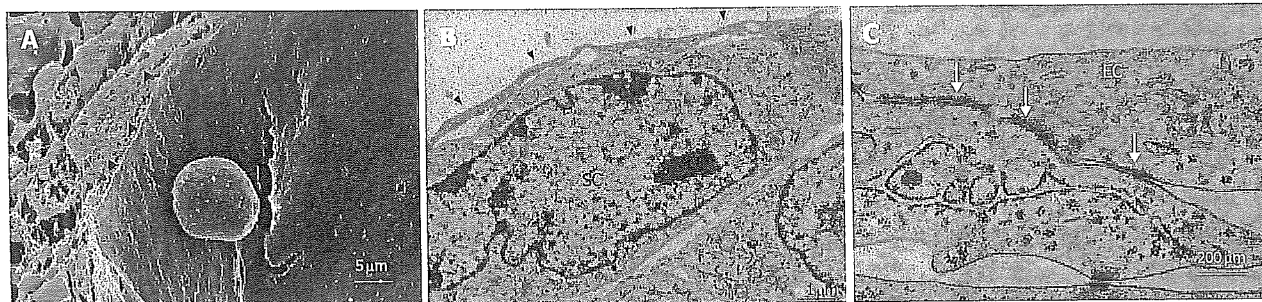


Figure 4 Ultrastructure of sinusoidal endothelial cells. A: Scanning electron microscopic image of sinusoidal endothelial cells localized at the perfusion side. They form a thin layer (arrowhead), showing the typical appearance of a sinusoid-like vascular structure. Scale bar: 5 μm; B: Transmission electron microscopic image showing sinusoidal endothelial cell growth at the perfusion side forming a thin layer (arrowhead) overlying the A7 cells (SC). Scale bar: 1 μm; C: Transmission electron microscopic view showing tight junctions (arrow) between sinusoidal endothelial cells (EC). Scale bar: 200 nm.

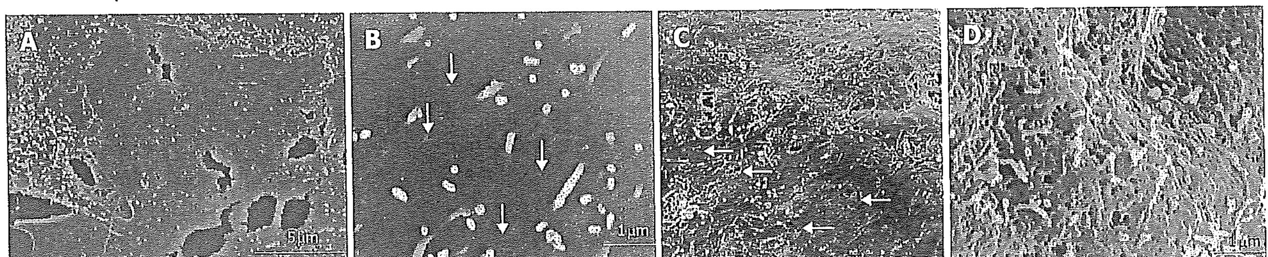


Figure 5 Scanning electron microscopic image of the surface of sinusoidal endothelial cells. A: Low-magnification scanning electron microscopic images of the surface of sinusoidal endothelial cells cultured on plastic dishes. The sinusoidal endothelial cells formed a thin layer on the plastic dish substrate. Scale bar: 5 μm; B: High-magnification scanning electron microscopy images of the surface of sinusoidal endothelial cells cultured on plastic dishes. Fenestrae could not be detected on the surface of endothelial cells. Only small pits are seen (arrow). Scale bar: 1 μm; C: Low-magnification scanning electron microscopic view of the surface of sinusoidal endothelial cells cocultured in the RFB. Fenestrated pores could be observed (arrow). Scale bar: 5 μm; D: High-magnification scanning electron microscopic view of the surface of sinusoidal endothelial cells cocultured in the RFB. Pores have a diameter of 100 - 200 nm. Scale bar: 1 μm.

(Figure 3A). TEM showed that cocultured cells assumed layered form from cellulose beads to the perfusion side (Figure 3B). M1 and A7 cells containing vitamin A-laden fat droplets were seen mainly at the perfusion side, while dense layers of FLC-5 cells were observed beneath (Figure 3C). At sites where the three cell lines were in contact with each other bile canaliculus-like structures were present between neighboring FLC-5 cells. Lumens of these structures contained electron-dense bile components, tight junctions and desmosomes also could be observed (Figure 3D). This side showed growth of endothelial cells with the formation of sinusoid-like vascular structures (Figures 4A and 4B). Tight junctions were seen between endothelial cells (Figure 4C). Fenestrae which are characteristic of SEC *in vivo*, were absent in monocultures of M1 cells on plastic dishes (Figures 5A and 5B). Because a long time subculture would change the character of M1 cells, pores were represent on the surface of M1 cells co-cultured in the RFB system (Figure 5C). The pores had a diameter of 100 to 200 nm, being similar in morphology and size to those of fenestrae shown by SEC *in vivo* (Figure 5D).

Morphology of M1 cells incubated with swinholide A

Cells incubated for 2 h with 200 nmol/L of the actin-disrupting agent swinholide A showed the increased number of pores (Figure 6), while some pores were dilated (about

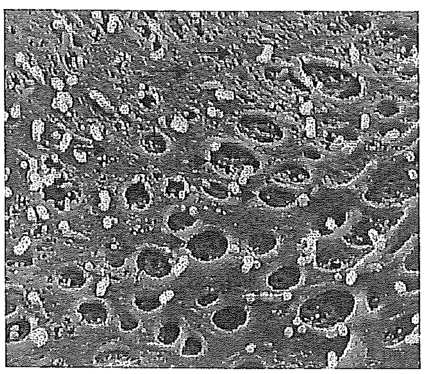
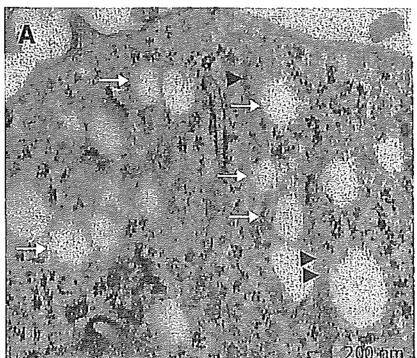


Figure 6 Scanning electron micrographs of the surface of swinholide A - treated SEC cells in the RFB culture system. Large open pores have a fenestra-like appearance (short arrow). Small pores were detectable in the nonfenestrated area (long arrow). Scale bar: 1 μm.



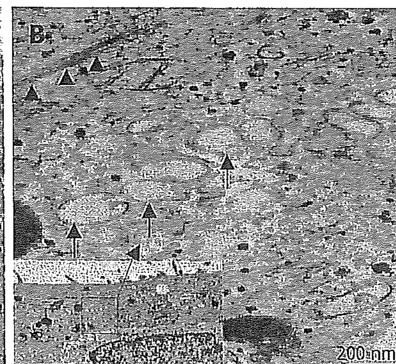


Figure 7 Transmission electron micrographs of sectioned SEC cells after swinholide A - treatment; A: Numerous open pores or fenestrae in the cytoplasm (arrow). Fine cytoskeletal elements showing a close spatial relationship with these pores (arrowhead). Scale bar: 200 nm; B: VVO could be observed in SEC cells (arrows) in response to stress or actin fibers (arrowheads). Scale bar: 200 nm. Inset shows the overall composition of the cells. Scale bar: 1 μm.

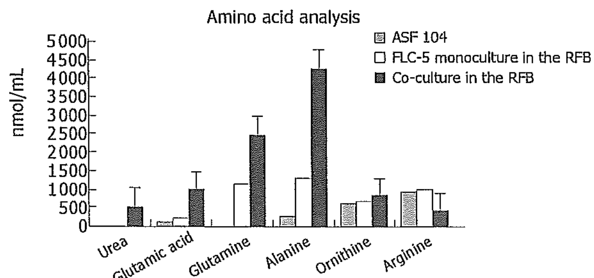


Figure 8 Amino acid and urea analysis in supernatants. Urea was detectable only in coculture, at 523 nmol/ml. ASF 104 designates culture medium. Mean value ± SD.

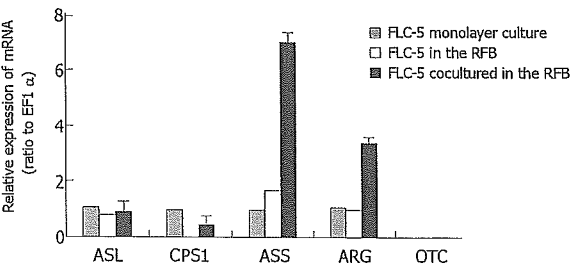


Figure 9 Comparison of expressions of CPS1, OTC, ASS, ASL, and ARG mRNA in FLC-5 incubated under different conditions as assessed by TaqMan 1-step RT-PCR. The mRNA expression of each enzyme in different conditions is relative to that in monolayer cultures. Mean value ± SD.

1 μm). Small pores (tens of nanometers in size) that probably resembled coated pits were abundant in the nonfenestrated areas.

TEM investigation showed that treatment with swinholide A resulted in fenestrated pores with a diameter between 100 and 200 nm. The pores fused with each other formed labyrinthine structures (Figure 7A). In addition, vacuoles with a diameter of about 200 nm, similar to previously described vesiculo vacuolar organelles (VVO), were noted. These structures typically were seen in areas where relatively regular overlap was seen in FLC-5, A7, and M1. The number of VVO increased when cells were treated with 200 nmol/L swinholide A, which was associated with partial fusion (Figure 7B).

Amino acid fractions from supernatants

At the end of culture, the supernatant was subjected to amino acid analysis. Urea production was not seen in monocultures of FLC-5 cells in the RFB, while FLC-5 cells cocultured with M1 and A7 cells produced 523 nmol urea /mL in the culture medium, suggesting that the urea cycle was activated in the coculture RFB system (Figure 8). Several amino acids were increased in the medium.

We compared mRNA expression of CPS1, OTC, ASS, ASL, and ARG in FLC-5 monolayer cultures with those of monocultures in the RFB system. In addition, mRNA expression in cocultures in the RFB also was assessed. We could not detect OTC in any type of culture. Expression of other urea cycle enzymes showed no notable difference between monolayer culture of FLC-5 and monoculture of FLC-5 in the RFB. However, ASS and ARG expressions in co-culture in the RFB were about 7 and 3 times greater than those in FLC-5 monolayer culture (Figure 9).

Albumin synthesis and expression of nuclear factors

We compared mRNA expression of albumin and HNF-1 and HNF-4 as transcription regulation factors between experimental conditions. Expression of mRNA encoding the three proteins was less in FLC-5 co-cultures in the RFB system than in FLC-5 RFB monocultures or in FLC-5 cells in monolayer culture (Figures 10A-10C). In a previous study, albumin production was enhanced in the RFB using the immortalized cell line[18]. However it was different cell line in this study.

DISCUSSION

Introduction of a functional human hepatocellular carcinoma cell line (FLC) in our system can allow the cells to be cultured at high density in a layered array and maintain viability for long periods[18,19].

Immortalized cells can be used for artificial liver. The reason is that it can supply cells in large quantities and quickly. Immortalized cells lose several characteristics in

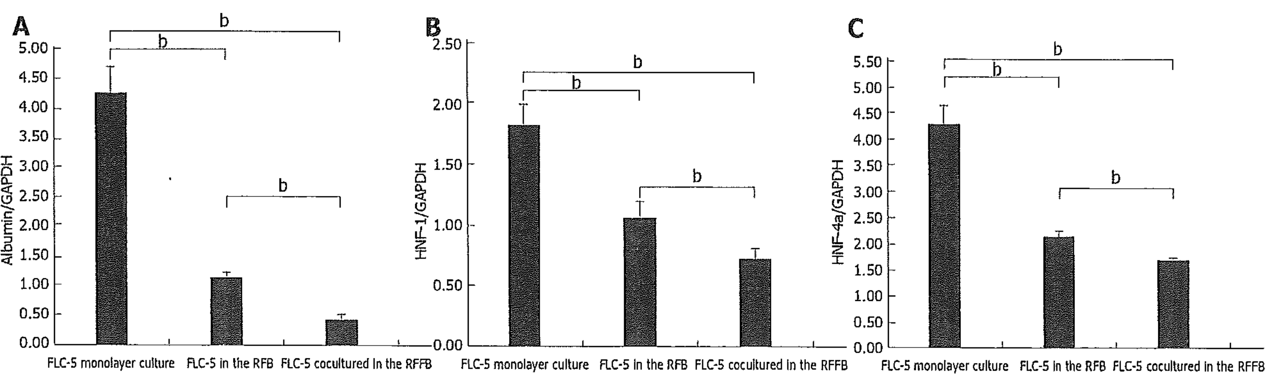


Figure 10 Expression of mRNA for albumin (A), HNF-4 (B) and HNF-1 (C) as transcription regulation factors in each condition. Messenger RNA expression for these three proteins decreased in cocultured FLC-5 in the RFB compared with FLC-5 monocultures in the RFB and FLC-5 cultured in a monolayer. Mean value ± SD. The ratio of mRNA for each protein versus GAPDH is shown. Differences with respect to each condition were statistically significant ($^bP < 0.01$) according to Student's *t*-test.

morphology and function. However, three cell lines were studied in our RFB culture system, including their fine structure according to electron microscopy. Layers of FLC-5, A7, and M1 were arranged respectively from the carrier attachment side to the perfusion side. In some areas, liver-like architectures, sinusoid-like lumen structure, bile-canaliculi and functional complex, were observed, comparable to *in vivo* tissue relationship. The M1 cell line well covered the perfusion side, mimicking vascular structures, indicating that this cell type forms an arrangement similar to that *in vivo*. Furthermore, M1 cells in monolayer culture did not express fenestrae, probably reflecting a long culture or subculture time[20]. In a previous study, we found that M1 cells also lack fenestrae in monocultures in the RFB[8]. In contrasts, fenestrated pores were seen in M1 cells cocultured according to the present RFB experimental design. Coculture and cell to cell contact have an influence on these morphological changes. Because fine structures *in vivo* could be observed better than monoculture in the RFB.

The electron microscopic observations in the present study clearly showed that if an appropriate environment for cell growth was provided in a perfusion culture system, the individual cell types could arrange themselves according to their *in vivo* characteristics, even in a high-density layered culture.

This study also examined the numerical dynamics of fenestrae. For this we exposed the cocultures to the actin-disrupting drug swinholide A[14]. When the cells were treated with swinholide A, the number of pores with a diameter of about 100 to 200 nm increased 2 h after swinholide A treatment. Furthermore, by TEM, cytoplasmic vesicles about 200 nm in diameter could be seen and were much larger than the caveolae in the cytoplasm, and their number increased in the presence of swinholide A. These vacuolar-like vesicles probably represent the vesiculo vacuolar organelle (VVO) as described by Feng *et al*[21]. The VVO is an organelle contributing to transport of macromolecules between luminal and abluminal sides of endothelial cells, thus increasing transcellular permeability. Vascular permeability factor and vascular endothelial growth factor (VPF/VEGF) can induce formation of VVO[22]. FLC-5 used in this study, could express VPF/VEGF (data not shown). The presence of vascular factors may partially explain why VVO is noted in cocultures and why fenestrae could be observed in our experiments[23]. VVO is thought to be formed by fusion of caveolae, when multiple VVOs fuse together, a structure extending from the luminal to the abluminal sides of endothelial cells is formed. In the present study, fused VVOs also were seen in swinholide A - treated specimens by transmission electron microscopy, suggesting that this fusion represents a process culminating in formation of the labyrinthine structures in SEC[24]. The mechanism of pore formation in immortalized SEC and under cocultured perfusion conditions remains unknown from the present study. However, pore formation may result from multiple effects or factors working in concert upon endothelial cells, such as cytoskeletal dynamics represented by actin and/or the influence of a yet unknown factor secreted by other cell types present in the cocultures such as VEGF. The observation that hepatic endothelial cells maintain one of their typical morphological features (i.e. an abundant number of membrane-bound coated-pits, uncoated vesicles/vacuoles and fenestrae) is an indication that the bioreactor mimics a nearby physiological cultivation environment for the various liver cell types. However, the mechanism by which the bioreactor and its culture environment bring about and maintain these membrane-bound vesicles and fenestrae in endothelial cells remain to be elucidated and consequently open up new directions for future experiments.

To assess hepatocyte function, we compared mRNA expression for urea cycle enzymes and albumin synthesis by FLC-5 in monolayer culture compared to these single-type cultures and cocultures in the RFB. Previously, we have demonstrated hepatocyte functions such as albumin synthesis and cytochrome expression are enhanced in the RFB[25, 26]. Urea production is among the most primitive functions of liver cells. We could not detect urea in medium from monolayer cultures or monocultured FLC-5 in the RFB. In contrast, FLC-5 cells cocultured in the RFB exhibit ability to produce urea, and mRNA expression for ASS and ARG is enhanced. The medium used in this experiment, ASF 104 contained arginine, so urea production was observed in cocultures in the RFB although OTC was not expressed. One report showed that urea produc-

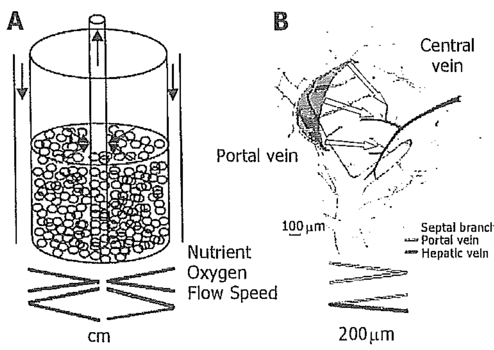


Figure 11 RFB and intact organ. **A**: In the RFB system, culture medium flows from outside the column toward the center of the reactor. Medium flows faster at the center than at the periphery. Biases in distribution of oxygen and nutrition at inflow and outflow are minimized. **B**: In the hepatic lobe, blood flows from the portal vein to central vein. The RFB system is similar to the organization of the hepatic primary lobe[31]. Figure 11B is reproduced from Figure 9 in reference 31.

tion in OTC-deficient mice could be detected under the same condition[27]. Glutamic acid, glutamine and alanine were also increased in supernatant co-cultured in the RFB, indicating that amino acid metabolism becomes active.

It was reported that three-dimensional spherical culture induces albumin synthesis, a particularly important hepatocytic function[28, 29]. However, in the present study, mRNA expression of albumin was decreased under coculture conditions in the RFB. Nuclear transcriptional factors HNF-4 and HNF-1, which regulate albumin synthesis, were decreased under coculture conditions in the RFB. Albumin in supernatant was also decreased during culture (data not shown). The results suggest that the culture environment (cell-to-cell communication, cell polarity, shear stress, and other factors) can control manifestations of intracellular nuclear transcription factors and therefore dramatically influence albumin production by liver cells. Immortalized cells can be used for artificial liver. The reason is that it can supply cells in large quantities and quickly. But immortalized cells may change the characteristics of its original cells. In this study, albumin synthesis was decreased. It was not useful for artificial liver. In future study, we have to try other cell sources (ES cell, oval cell, and other immortalized cell lines).

Finally, several points should be noted concerning our culture system. First, controlling the mixture ratio of the three cell types used is very difficult since each type possesses its own potential for active growth. Thus, growth rates vary between cell types and are difficult to control. For examples, A7 cells grew less rapidly and tended to be less than the other two cell types in the coculture system. Second, the hepatic lobule spans about 140 μm *in vivo*, extending from the portal to the central area, toward which portal blood flows in a radial manner. According to Matsumoto *et al*[30], the liver is an organ composed of numerous groups of microscopic three-dimensional units (minimal radial-flow bioreactors) extending from the inflow side (composed of combinations of parabola-shaped inflow fronts) to the central vein[31]. According to this model, the liver microcirculation as observed *in vivo* could not be reproduced faithfully with a radial-flow bioreactor, since the distance between inflow and outflow sides in the bioreactor is about 1.5 cm (Figure 11). Third, bile canaliculus-like structures are formed between hepatocytes. Since we did not use the bile duct cells in this study, whether different cell types can reconstruct bile ducts remains to be elucidated[32]. Finally, although several questions remain, the results of the present study suggest that liver reconstruction is possible *in vitro*. Such organ reconstruction technology is expected to contribute greatly to the development of sophisticated artificial livers and other organs for transplantation. Our culture system may be a very important tool to maintain liver organ.

ACKNOWLEDGMENTS

The authors thank Mr. Hideki Saito, Mrs. Emi Kikuchi, and Mrs. Hisako Arai of the DNA Medical Institute at The Jikei University School of Medicine for technical assistance with electron microscopy. The authors also thank the members of the Australian Key Center for Microscopy and Microanalysis of The University of Sydney for their excellent administrative, technical, and practical support.

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S- Editor Wang J **L- Editor** Wang XL **E- Editor** Liu WF

REVIEW ARTICLE

Bioreactors for 3-dimensional high density culture of human cells

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Abstract

A bioreactor was developed as an instrument to culture human or animal cells that require attachment in a large quantity or at a high density. The purpose for developing such a bioreactor is two-fold: to produce a large quantity of animal or human cells that have been modified by gene recombination technology to accommodate manufacture of physiologically-active substances or human proteins on an industrial scale; and for research to culture animal cells to form a high-density 3-dimensional structure as a morphological or functional tissue or organ entity. In the current report, the circulatory flow bioreactor and radial flow bioreactor (RFB) are introduced, in which the former can be scaled up. As a small bioreactor produced for the latter purpose, a rotary cell culture system and novel multicoaxial hollow-fiber bioreactor are introduced. Finally, a small RFB culture system that was scaled down by the present author and his collaborators for the study of a 3-dimensional high density culture system is described. The RFB can be readily scaled up for manufacturing or scaled down for research purposes. This is a cell culturing system that can induce the functions of human tissues by preparing a high density 3-dimensional organization of cells of human origin.

Key words: 3-dimensional culture, bioreactor, high density culture, organoid, protein production.

INTRODUCTION

An animal-cell culturing method using a bioreactor has been developed to mass produce cells for manufacturing physiologically-active substances on an industrial scale; and to culture high-density 3-dimensional masses of these cells for reconstruction of tissue and organ entities to improve their functions. The bioreactors for the latter purpose can also be used for the development of bio-artificial organs. The intent of this overview is to describe the bioreactors that are currently being developed and utilized for high-density 3-dimensional culturing of cells. Introduced at the end of this study is a radial flow bioreactor (RFB), which has been scaled down, adapted to a simpler high-density 3-dimensional culturing system and utilized for the development of a bio-artificial liver.

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Received 2 November 2005; accepted 12 November 2005.

BIOREACTORS FOR INDUSTRIAL PRODUCTION

A bioreactor for industrial production was developed to culture as many cells of animal origin as possible to produce the desired preparation. Unlike yeasts and coliform bacteria, many animal cells cannot be maintained while suspended in a fermentation vat: they require attachment for growth. The pharmaceutical industry engages in the commercial drug production of physiologically-active substances that are normally produced in minute quantities (e.g. interferon, erythropoietin and G-CSF). Many of the bioreactors that are used for this purpose rely on the roller bottle system.[1] In practice, many large culture bottles are arranged in a large-scale

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H U C	0 0 2	Operator: Zhao Xianglan	Dispatch: 07.01.06	PE: Marko Stechiwskyj
Journal Name	Manuscript No.	Proofreader: Liao Bo	No. of Pages: 6	Copy-editor:

pharmaceutical-company facility that is operated under strict safety controls to conform to Good Manufacturing Practices (GMP) and cells are cultured using a large quantity of a culture fluid. The substance that is produced by these cells and released into the culture fluid is extracted, purified and turned into the desired preparation. To increase the culture yield, microcarriers measuring 100–200 μm in particle diameter and having a specific gravity of 1.03–1.05 are added to the fermentation vat as a carrier of the cultured cells.[2] In this manner, the surface area to which the cells attach themselves is substantially increased. The physiologically-active substances, such as those noted above, are given to a patient in trace amounts, i.e. in the order of μg in the form of a pharmaceutical product. Thus the conventional roller bottle system is adequate for the production of active substances in minute quantities. Currently however, a variety of antibody preparations (represented by a human-mouse chimera monoclonal antibody against mammary cancer HER2, trastuzumab [Herceptin, Chugai-Roche]) have been developed and are expected to become the mainstay of the pharmaceutical industry.[3] The dosage of these antibody preparations is administered in the order of mg to a patient. Thus the need to design a highly efficient mass production system to accommodate the manufacture of antibody preparations was recognized. Furthermore, it will become necessary in the near future to develop a production system for plasma products, such as albumin and several coagulation factors that have to be measured in grams and added to the actual production line. Many researchers believe that yeasts and coliform bacteria may be used instead of human cells to mass produce these human proteins. It is true that from a cost aspect, serial culture systems (such as those using yeast cells) are most efficient for producing substances that utilize gene recombination technology. But no matter how thorough efforts may be in the purification process to remove impurities, such as yeast proteins, the increase in the dosage normally given to a patient will inevitably result in contaminating the product with a small amount of foreign proteins. This minute quantity of impurity may cause severe allergic reactions, such as an anaphylactic shock. For this reason, a bioreactor is needed that enables one to culture a large quantity of human cells at a high density, thus manufacturing human protein products as inexpensively and safely as possible.

A bioreactor that has already been in use on a production line is a circulatory flow type developed by Meiji Dairies Corporation (Fig. 1).[4] One may consider this to be a type of bioreactor that enables culturing in an improved fermentation vat or simply a fluid-type bioreactor. Animal cells that proliferate at rates much slower than yeasts and a production system that relies on a batch process are associated with very poor efficiency. Therefore various innovations have been made to improve productivity, through repeated batch processing, fluid culturing and serial culturing. In this bioreactor, productivity is improved by sealing the animal cells in the system at a high density to eliminate the time required for proliferation, supplying the medium serially and extracting the product. To maximize the density of the cells to be cultured, a gas exchange chamber is located at the upper section of the vat; the culture fluid and the gas (a mixture of oxygen and air) are agitated by a rotor within the vat; and the resultant fluid is circulated in the section where cells are cultured to supply sufficient oxygen and nutrients. A folded glass-fiber cloth fills the section where the cells are cultured to provide an attachment carrier for these cells and maximize the cell density. The Meiji Dairies Corporation prepared 50-L vats. They successfully cultivated those cells producing the HBs antigen in a high quantity (huGK-14 cells) that had been cloned from Huh1 cells. This company manufactures and markets the HBs antigen protein that precipitated as a type B hepatitis vaccine.

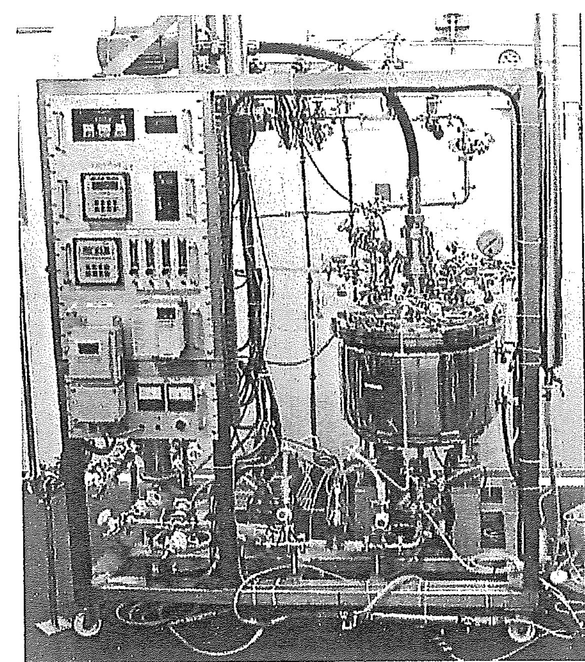

Figure 1 The circulatory flow bioreactor developed by Meiji Dairies Corporation.

The radial flow bioreactor (RFB), a cell-filling type bioreactor that was developed by Central Laboratories for

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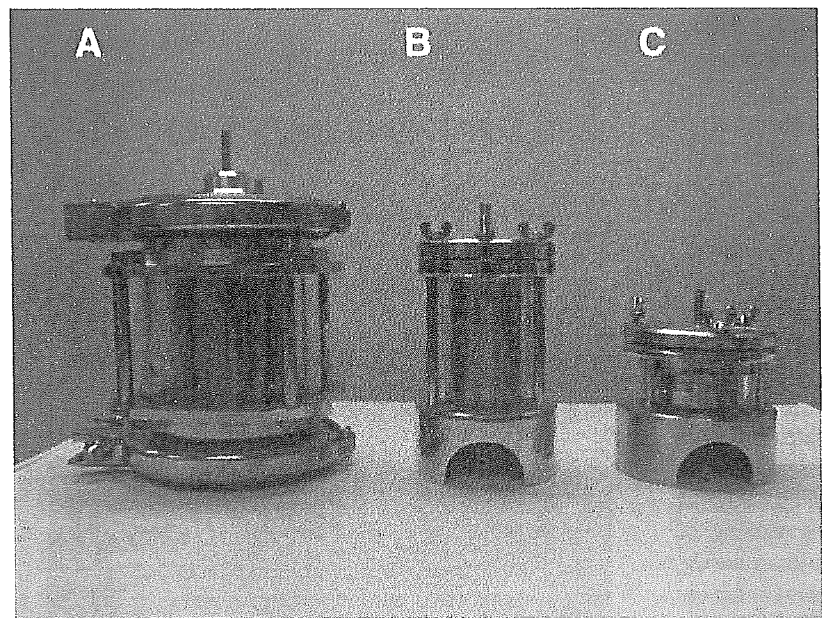

Figure 2 The radial flow bioreactor (RFB) developed by Kirin Brewery Co. Ltd. (A) 200-mL-volume RFB; (B) 50-mL-volume RFB; (C) 15-mL-volume RFB.

Key Technology of Kirin Brewery Co., Ltd, raises the density of the cells being cultured to the absolute maximum and increases the protein productivity more than 100 times that of the roller bottle culture system[5] (Fig. 2). The columnar bioreactor is filled with the cell carriers for seeding and attaching the cells, while the culture fluid is radially circulated from the periphery of the column to the central section. The deviations in the nutrient and oxygen concentrations at the entry and exit of the culture fluid that are caused by high-density cultivation are adjusted by the reflux velocity associated with the circulation in the direction toward the center of the column. Kirin claims that high-density culturing at a volume of 1×10^8/mL, which is the highest ever achieved by any bioreactor, is possible. As its prototype, a bioreactor with a total capacity of 2.8 L and the capacity of the section (to be filled by the carrier) of 0.9 L has been fabricated and operated. Theoretically, it is possible to increase the capacity to 67 L. It is a very promising bioreactor for human and animal cells culturing to produce desired products.

BIOREACTORS FOR RESEARCH PURPOSES

For research, many bioreactors have been developed for diverse purposes, which include: (i) studies of infections (HIV, Ebola virus infection, Lyme disease, and HCV); (ii) cancer models (involving prostate, breast, ovary, lung and colon); (iii) research on diabetes mellitus; (iv) therapies for musculoskeletal tissue disorders (in space and on earth); (v) drug efficacy (nonanimal/nonclinical testing of drug effects and toxicity); and (vi) development of bio-artificial organs. These goals cannot be met by simply culturing a large quantity of cells: the animal cells must be cultured as 3-dimensional clusters forming cellular polarities. In other words, these bioreactors are devices to reconstruct tissue and organs from cells. The conditions to achieve such an end include: (i) correction of deviations in the oxygen and nutrient concentrations; (ii) removal of waste products; (iii) optimum shear stress; and (iv) adjustment of cell precipitation caused by gravity.

Among the bioreactors that have been developed, only a limited number of them meet these conditions. For an all-purpose research reactor, the rotary cell culture system (RCCS) that has been developed by NASA is cited first. Commonly called a rotating wall vessel bioreactor (RWV), it is manufactured and marketed by Synthecon Inc., Houston, TX (it is handled by TOMY Digital Biology Co., Ltd. in Japan, http://www.digital-biology.co.jp) (Fig. 3).[6] Originally, the reactor was characteristically rotated in the weightless environment of a spaceship to create microgravity with a scenario in which animal cells are cultured in a 3-dimensional form. This concept is also effective on the earth's surface. The columnar reactor (vessel) is laid horizontally and filled with the culture fluid. Its axis is slowly rotated, generating an average microgravity of $10^{-2} \times g$. Thus the cells in the RWV are

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Figure 3 The rotary cell culture system (RCCS) developed by NASA. It is handled by TOMY Digital Biology Co., Ltd. (http://www.digital-biology.co.jp) in Japan. (A) 3-dimensional cell culture system for one vessel (RCCS-1); (B) 3-dimensional cell culture system for four vessels (RCCS-4).

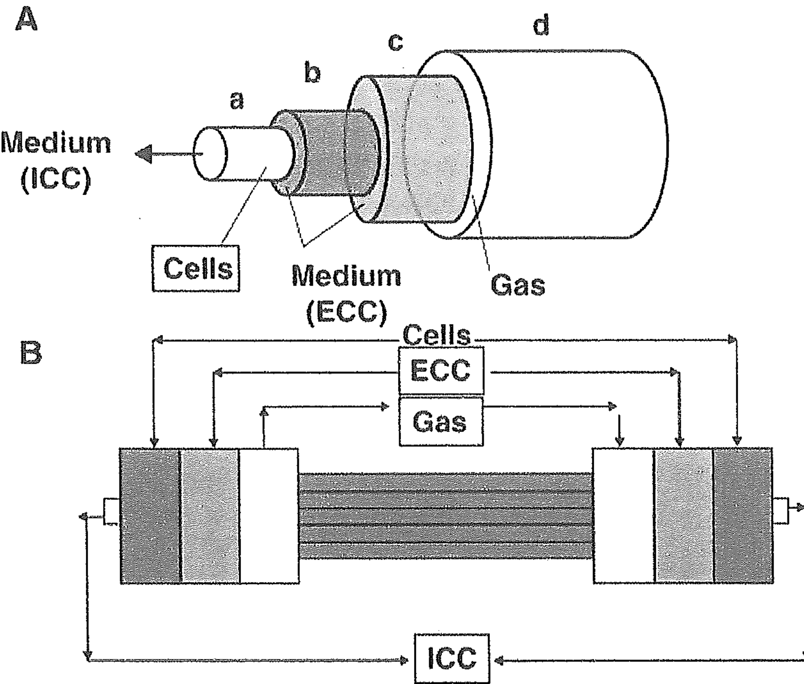

Figure 4 A novel multicoaxial hollow-fiber bioreactor (schema). (A) The three concentric tubes and housing tube: a, inner hollow fiber; b, middle hollow fiber; c, silicon tube; d, housing tube. (B) three major compartments of the multicoaxial bioreactor. The ECC and ICC reservoirs are on the top and bottom of the flow diagram.

kept from being exposed to gravity as high as 1 × g. This result in cellular aggregation, subsequent intercellular adhesion, and gradual formation of 3-dimensional cell clumps. Of the conditions for 3-dimensional culturing cited above, the RWV satisfies "(iii) optimum shear stress" and "(iv) adjustment of cell precipitation caused by gravity." In fact, there is a report on the successful formation of a liver "organoid" measuring 1–3 cm in length by combination with a microcarrier.[7] The device is also suitable for coculturing and tissue explanting, allowing observation of the manner by which cancer cells adhere and infiltrate the host tissue. Rotary Culture Max, a large culture system for preparing large 3-dimensional tissue architectures and organoids, has also been developed.

The conventional hollow-fiber bioreactor has been plagued by a problem: the uneven distribution of oxygen and nutrients, which disturbs high-density culturing. Therefore it was difficult to scale-up with this device. The novel multicoaxial hollow-fiber bioreactor, originally developed to prepare a bio-artificial liver, was designed to overcome this problem (Fig. 4).[8] The device is composed of a combination of two tubes and two hollow fibers; a housing tube composed of a polycarbonate that does not permit gases to permeate from outside; a silicon tube with

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good gas permeability but with no liquid permeability; a hollow fiber (middle hollow fiber, MHF) measuring 1 mm in diameter and having 0.2-μm inside; and a hollow fiber (inner hollow fiber, IHF) measuring 1 mm in diameter with 0.65-μm pores further inside. The cells are cultured in the space between the MHF and IHF or on the surface of these hollow fibers. The culture fluid is supplied to the space between the silicon tube and MHF; and oxygen- and carbon dioxide-mixed gases are brought in from the outside. Through pores measuring 0.2 μm of the MHF, the culture fluid flows radially to the cells, then to the IMH through its pores (measuring 0.65 μm) and is extruded. The capability of this bioreactor in culturing at a high density and the extent of its scaling up cannot be ascertained from the literature; but it is interesting because of the improvement that was made over the uneven distribution of oxygen and nutrients in the conventional hollow fiber bioreactor.

SIMPLIFIED SMALL RFB CULTURE SYSTEM FOR EXPERIMENTAL STUDIES

The RFB cited earlier was originally developed in Japan for use as a cell-filled bioreactor to mass produce proteins. Nagamori *et al.* scaled down this design for the production of a bio-artificial liver.[9–12] The models developed for kinetic studies by Mizutani of Central Laboratories for Key Technology (now the Central Laboratories for Frontier Technology) of Kirin Brewery, Inc. include large reactors, ranging in capacity from 900 mL to as small as 400 mL. Nagamori *et al.* created a 100–30 mL capacity RFB and cultured human hepatoma cells to be used as a bio-artificial liver in a preclinical experiment. The author and his colleagues cultured highly functional human hepatoma cells, FLC-4, in a 15 mL capacity RFB and created an extracorporeal type bio-artificial liver. In this experiment, an acute hepatic failure was induced in minipigs that weighed 10–15 kg. An experiment was conducted on extracorporeal circulation using this bio-artificial liver, which was found to be effective in averting the development of fatal hepatic encephalopathy. The RFB constituting the mainstay of the bio-artificial liver is operated by an extensive system composed of a large reservoir, oxygen electrode, thermometer, pH sensor, controller, personal computer and pump, all of which were incorporated into the extracorporeal circulatory system at the time of treatment. For a 3-dimensional high-density reflux culture test, the current author and his collaborators developed a small RFB culture system by further reducing the size of the RFB to a 5 mL capacity and simplifying the peripheral equipment (Fig. 5). To prevent the cells from precipitating by gravity, the culture fluid was designed to circulate upward from the lower section of the bioreactor. The reactor was filled with

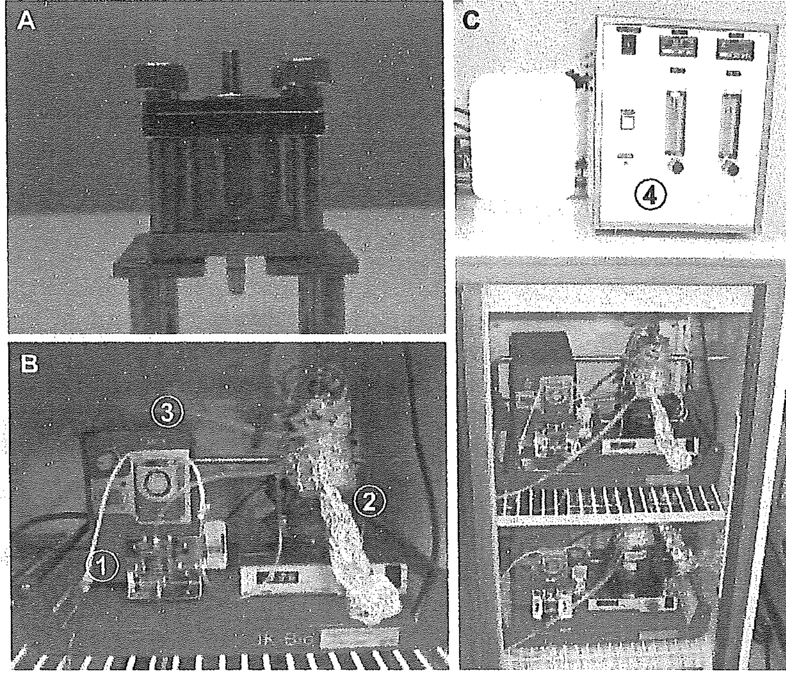

Figure 5 The mini-RFB culture system. (A) 5-mL-volume RFB; (B) Bioreactor system: 1, mini-RFB; 2, reservoir; 3, circulation pump. (C) Two mini-RFB culture systems supplied CO_2–air mixture gas; 4, a mixed gas feeder.

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porous hydroxyapatite beads measuring around 1 mm in diameter (PENTAX, Tokyo). The culture fluid enters from the lower part of the reactor, is refluxed radially from the periphery toward the center and leaves at the lower section. The most prominent innovation of this small RFB culture system is the device to supply a CO_2–air gas mixture. Initially, the entire system was placed in a CO_2 incubator to operate but it was soon found out that when cells have proliferated, the culture fluid tends to become acidic in the 5% CO_2–air mixture and the product does not function well as an organoid. To fix this, a device was created in which the CO_2–air mixing ratio was manually reduced and the gas mixture was supplied at a fixed (20 mL/minute) flow volume (patent pending, JK Bio, jkbio@hotmail.co.jp). By this means, it became possible to culture cells in multiple small RFBs under identical conditions. A high-density 3-dimensional culture system permits one to observe the process of enzyme (CYP3A) induction for the first time, which was not possible in a single layer culture.[13] CYP3A is induced *in vivo* by drugs such as rifampicin, constituting a major cause for drug interactions. The CYP3A4 enzyme is induced when pregnane X receptor (PXR), a nuclear transcription factor, binds the retinoid X receptor (RXR) to form a heterodimer in ER6 of the promoter region of CYP3A4 gene. It has been proven that when human hepatoma cell FLC-5 undergoes a high-density 3-dimensional culturing process in the RFB, this heterodimer further binds some nuclear protein to form a larger complex and CYP3A4 is more intensely induced. It became evident that by employing a 3-dimensional high-density culture technique, enzyme reactivity occurs that simulates the process taking place in the hepatic tissue *in vivo* more closely than any of the conventional systems. These observations suggest that this bioreactor may be useful in the study of reconstruction of not only the liver but also various other tissues, such as the kidney and pancreas.

CONCLUSION

The radial flow bioreactor was developed as a reactor – theoretically scaled up to 67 L – for high-density culturing and the production of biological products. It was currently scaled down to a volume of 5 mL and an improved small bioreactor system that one can use for research without cumbersome procedures was produced. It is a very versatile bioreactor that may be used to reconstruct human organs and tissues from human cells for the study of virus infections, developing cancer models, diabetic investigations, and pharmacological research related to drug metabolism, toxicity, and interactions, and bio-artificial organ development. It is earnestly hoped that many researchers take advantage of this cutting edge biotechnology of which Japan can be proud.

ACKNOWLEDGMENTS

I thank to Meiji Dairies Corporation and TOMY Digital Biology Co., Ltd. for a precious photographic grant. This study was supported in part by grants-in-aids from the University Start-Ups Creation Support System at 2002–04.

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発行日　　平成 18 年 4 月
発行者　　大　川　　清
印刷所　　有限会社トック
〒105-0003
東京都港区西新橋 3-9-5
03-3431-1311

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