Jikeikai Med J 2005 ; 52 : 109-14

Two-Dimensional Protein Analysis of Functional Liver Cells For Bioartificial Liver

Tomasz HASCILOWICZ[1], Makoto KOSUGE[1,2], Tomokazu MATSUURA[3], Senya MATSUFUJI[1], and Noriyuki MURAI[1]

[1]*Department of Biochemistry (II), The Jikei University School of Medicine*
[2]*Department of Surgery, The Jikei University School of Medicine*
[3]*Department of Laboratory Medicine, The Jikei University School of Medicine*

ABSTRACT

A viable and functional hepatic cell line is required for the development of an efficient bioartificial liver. Cell lines that have been derived from human hepatocellular carcinoma (FLC4, FLC5 and FLC7) maintain several liver-specific functions and can be grown in the radial flow bioreactor (RFB), which allows large-scale, high-density, and long-term culture, and is thus promising for use in a bioartificial liver. In the present study proteins secreted by FLC4, FLC5, and FLC7 cells grown both in monolayer and in RFB culture conditions were analyzed with two-dimensional polyacrylamide gel electrophoresis. The protein spots were assigned with mass spectrometry and by comparison with a public proteome database. The secretory protein profiles indicated that all cells grown either in monolayer or in RFB cultures secreted several physiologically important liver-specific proteins, although the proportions varied among cell lines and between culture methods. The results will be useful as basic data for selecting appropriate cells for the clinical and experimental use of bioartificial livers. (Jikeikai Med J 2005 ; 52 : 109-14)

Key words : bioartificial liver, hepatocyte, radial flow bioreactor, two-dimensional polyacrylamide gel electrophoresis, secretory protein

INTRODUCTION

The bioartificial liver is a device containing functional hepatocytes that can provide temporary support of hepatic function for patients with liver failure[1]. Expected applications of the bioartificial liver include temporary liver support for patients with severe, acute liver failure and serving as a bridge to liver transplantation[2]. Various types of bioartificial liver have been developed and can be categorized by the type of the culturing apparatus (bioreactor) and by the functional cells grown within[1]. Two types of bioreactor are widely used : three-dimensional bioreactors that contain suitable carriers in the reactor chamber for adhesion of the cells, and hollow-fiber bioreactors that contain stacked thin fibers within which cells attach. The radial flow bioreactor (RFB) is a sophisticated three-dimensional bioreactor that allows large-scale, high-density, and long-term cultures of functional cells[3]. The RFB is a cylindrical shell filled with porous microcarriers through which liquid medium flows from the periphery toward the central outlet tube, providing a beneficial concentration gradient of oxygen and nutrition within the medium and preventing accumulation of waste products or excessive shear stress.

The choice of cell is a critical factor for the effectiveness and practicality of a bioartificial liver

Received for publication, November 29, 2005
Tomasz Hascilowicz, 小菅　誠, 松浦　知和, 松藤　千弥, 村井　法之
Mailing address : Noriyuki MURAI, Department of Biochemistry (II), The Jikei University School of Medicine, 3-25-8 Nishi-shimbashi, Minato-ku, Tokyo 105-8461, Japan.
E-mail address : nmurai@jikei.ac.jp

system. A variety of cells have been used in trials of bioartificial livers in humans. These cells include normal human adult or fetal hepatocytes, cell lines established from hepatocellular carcinoma, hepatocytes that have been reversibly or irreversibly immortalized by transformation in vitro, cells derived from human stem cells, and hepatocytes of xenogeneic origin[1,2]. However, none of these cells are ideal for practical use in bioartificial livers because of concerns over safety, stable supply, and ethical issues.

The use of well-characterized human hepatocellular carcinoma cell lines has significant advantages. FLC4, FLC5, and FLC7 (previously known as JHH4, JHH5, and JHH7) are cell lines established from Japanese patients with hepatocellular carcinoma[4]. These cell lines are free from the hepatitis viruses, maintain several liver-specific functions, and can grow in the RFB[3]. The combination of these cells with an RFB has proven to be a useful experimental model for studying the morphology and regeneration mechanisms of hepatic organization[3], the kinetics of drug metabolism[5], and the propagation of hepatic viruses[4,6].

For clinical and experimental application of a bioartificial liver using FLC cell lines, the function of each cell line should be characterized. In the present study, we examined secretory functions of cell lines grown in three-dimensional culture in the RFB and compared these functions with those of cells grown in monolayer cultures on plastic dishes by means of analysis of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

MATERIALS AND METHODS

Cell cultures

The FLC4, FLC5, and FLC7 cell lines were maintained on 10-cm-diameter tissue culture dishes (Corning Japan, Tokyo) as described previously[3]. For monolayer cultures, confluent cells in the dishes were harvested with trypsin, diluted 20-fold in ASF104 serum-free medium (Ajinomoto, Tokyo), and seeded on 10-cm-diameter tissue culture dishes in 10-ml volumes. When cells reached a subconfluent growth stage, the medium was replaced with fresh ASF104 serum-free medium. All cultures were carried out at 37°C in a humidified 5% CO_2 atmosphere. For three-dimensional culture, FLC4, FLC5, and FLC7 cells were grown in an RFB culture system (reactor volume, 15 ml; Able, Tokyo) packed with hydroxyapatite ceramic beads (diameter, 1-2 mm; pore size, $<200\ \mu$m; Asahi Optical, Tokyo) with a constant flow of ASF104 serum-free medium for 10-14 days. The total volume of the medium in the system, including the conditional vessel and the tubing, was approximately 250 ml.

Preparation of protein samples

The media were collected for analysis from the cultures 3 days after the cells reached confluency for both the monolayer and the RFB culture conditions. This is because the saturated condition is considered to be closer to the physiological environment in the liver and better able to maintain the cells in a differentiated state than can a growing culture condition. The collected culture media, either fresh or having been stored at −80°C, were centrifuged at 1,500 rpm. The supernatants were concentrated 100- to 200-fold with a filtration apparatus (Amicon model 8400, Millipore Japan, Tokyo) and Ultrafree filter units (Amicon Biomatrix-5, Millipore Japan). Concentrated samples were then dialyzed against 0.2 mM Tris-HCl, pH 7.0. Protein concentrations were determined with the Bradford method with a kit (BioRad Japan, Tokyo) using bovine serum albumin as a standard.

2D-PAGE

2D-PAGE analysis was carried out according to the method of O'Farrell[7] with some modifications[8]. Briefly, protein samples (20 mg) were mixed with four volumes of the isoelectric focusing (IEF) sample buffer (5 M urea, 2 M thiourea, 1% ampholine, pH 4-10, 5% mercaptoethanol, 2% Nonidet P-40) and loaded on capillary IEF gels with a fixed pH range of 4 to 10 (IPG tube gel, Dai-ichi Kagaku, Tokyo). The IEF electrophoresis was started at 400 V for 30 minutes, after which the voltage was gradually increased to 1,000 V over one hour and maintained at 1,000 V for 15 hours. Before the electrophoresis was finished,

the voltage was raised to 1,500 V for 1 hour and then to 2,500 V for an additional hour. Isoelectric point markers (Broad pI Kit, Amersham Bioscience, Piscataway, USA) were run in parallel to indicate the pH positions. Following IEF electrophoresis, the capillary gels were equilibrated in sodium dodecyl sulfate (SDS)-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 4.3% SDS, 5% 2-mercaptoethanol, 30% glycerol, 0.01% bromophenol blue) and washed briefly twice in the SDS-PAGE running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS). The SDS-PAGE (the second-dimension electrophoresis) was performed on 184×185-mm 10% to 20% gradient gel plates (PAG Radi 2D, Dai-ichi Kagaku) at 75 mA for approximately 4 hours. Broad Range Protein Markers (New England Biolabs, Beverly, MA, USA) were used as molecular weight markers.

Assignment and quantification of protein spots

The 2D-PAGE gels were stained with the 2D Silver Staining Reagent-II (Dai-ichi Kagaku) and scanned with an ES-2200 scanner (Epson, Suwa). Major protein spots on each 2D-PAGE gel were initially assigned by comparison with the 2D-PAGE pattern of proteins secreted by a human hepatoma cell line, HepG2 cells, which is available from a public proteome database, SWISS-2DPAGE (http://au.expasy.org/ch2dothergifs/publi/hepg2sp.gif) (9). Next, several protein spots on the 2D-PAGE gel of RFB-cultured FLC5 were selected and confirmed by identification with MALDI-TOF mass spectrometry. The relative amounts of protein spots were calculated from their intensities relative to that of all spots for a given gel using Phoretix 2D software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

Mass spectrometry

For matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, 2D-PAGE gels were stained with Coomassie brilliant blue R25. Each spot on 2D-PAGE was excised and digested with trypsin and an In Gel Digestion kit (Promega, Madison, WI, USA). Digested samples were then concentrated and desalted with a Zip-Tip (Millipore Japan), a small disposable pipette tip with a bed of reversed-phase chromatography media fixed at its end. MALDI-TOF mass spectrometry was performed with an AXIMA-CFR mass spectrometer (Shimadzu, Kyoto). Obtained fragment ion patterns were analyzed with the Mascot Search Engine program (Matrix Sciences, Tokyo).

Results

Total protein concentration in culture media

Culture media were collected from monolayers and RFB cultures of FLC4, FLC5, or FLC7 cells. Protein concentrations in the media varied from 1.4 mg/l (RFB cultured FLC4) to 22.1 mg/l (monolayer cultured FLC7) (Table 1). The medium used under these culture conditions did not contain serum as an additive.

Identification and assignment of proteins on 2D-PAGE gel

Secretory proteins produced by FLC4, FLC5, and FLC7 cells grown in monolayer and RFB cultures were separated with 2D-PAGE (Fig. 1). We identified 4 protein spots as albumin, transthyretin, 1-antitrypsin, and transferrin. Other protein spots were assigned with Phoretix 2D software and spot position data of the HepG2 secretory protein pattern from the SWISS-2DPAGE. At least 10 additional protein spots were assigned as hemopexin, ceruloplasmin, α1-antichymotrypsin, α2-HS-glycoprotein, clusterin, serum retinol binding protein (SRBP), apolipoprotein A-I (ApoA-I), fibrinogen α chain, fibrinogen β chain, and complement 3α (C3α).

Table 1. Total protein concentration in culture media

Culture	Cell line	Protein concentration in culture medium (mg/l)
Monolayer	FLC4	7.3
	FLC5	11.5
	FLC7	22.1
RFB	FLC4	1.4
	FLC5	1.5
	FLC7	4.6

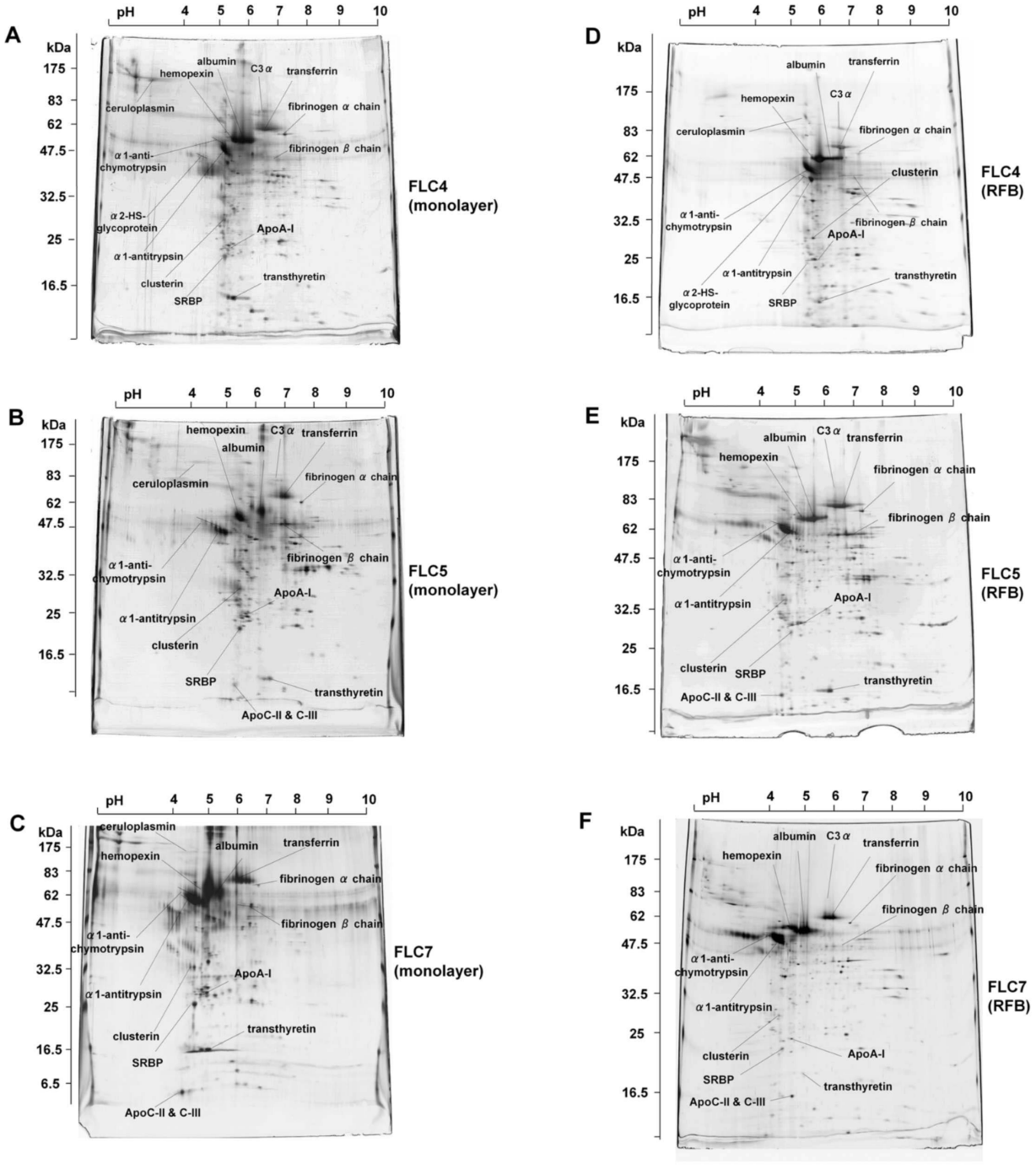

Fig. 1. 2D-PAGE analysis of proteins secreted by FLC4, FLC5, and FLC7 cells. Positions of molecular mass markers and isoelectric point markers are indicated by the vertical and horizontal scales, respectively. Protein spots were visualized with silver staining and assigned with MALDI-TOF mass spectrometry or by comparison with the HepG2 secretory protein database from SWISS-2DPAGE. Assigned spots are labeled. A–C, monolayer cultures; D–F, RFB cultures. A and D, FLC4; B and E, FLC5; C and F, FLC7.

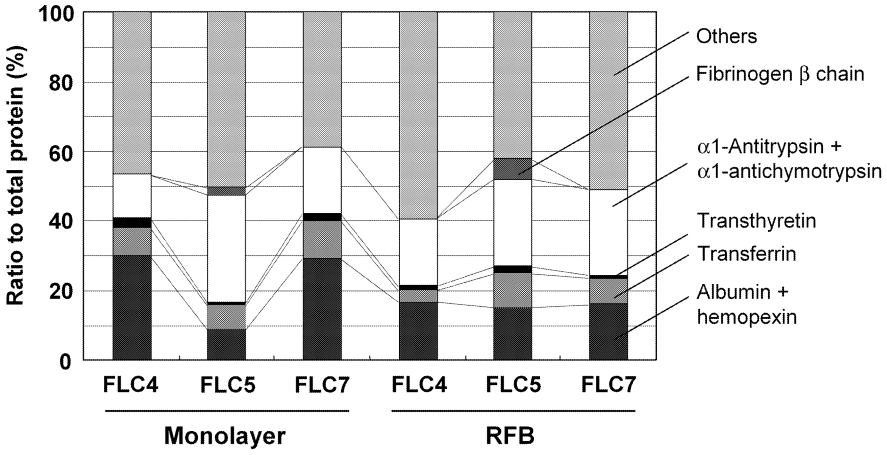

Fig. 2. Relative amount of major components in total secretory proteins. Calculated ratios of each protein spot/total protein are indicated. Albumin and hemopexin as well as α1-antitrypsin and α1-antichymotrypsin are often inseparable and expressed as the ratios of their sums.

Table 2. Relative amount of minor components in total secretory proteins

	Ratio to total protein (%)					
	Monolayer			RFB		
	FLC4	FLC5	FLC7	FLC4	FLC5	FLC7
Apo A-I	0.84	1.38	1.61	0.26	0.24	0.46
SRBP	0.10	0.00	0.34	0.13	0.13	0.03
Ceruloplasmin	0.30	0.14	1.53	0.45	0.46	1.29

Profiles of secretory proteins

The secretory proteins produced by the three cell lines grown under either culture conditions were quantified. The relative amounts of major secretory proteins in total secretory proteins were shown in Fig. 2. The relative amounts of some of the minor secretory proteins were shown in Table 2.

Discussion

Bioartificial livers rely on the hepatic functions of the employed cells, such as detoxification, metabolic function, and synthesis of proteins and other molecules. In the present study, we investigated proteins secreted by the human hepatocellular carcinoma cell lines FLC4, FLC5, and FLC7, which have been established for use in bioartificial livers.

Concentrations of proteins were higher in the media from the monolayer cultures than in the media from the RFB cultures (Table 1). This result, however, does not necessarily mean that RFB-cultured cells have a lower capacity for protein secretion, because the total medium volume per cell and the interval of medium replacement differed between the monolayer and RFB culture. Of the three cell lines, FLC7 yielded culture medium with the highest concentration of proteins under both culture conditions.

Quantification of protein spots revealed the secretory protein profiles of the cell lines and culture conditions. The ratios of major proteins varied less among the cell lines in the RFB culture than in the monolayer culture (Fig. 2). The ratios of minor secretory proteins, however, varied more among the cell lines than did the ratios of major proteins. For example, secretion of fibrinogen β chain was only observed in FLC5 (Fig. 2), and FLC7 secreted SRBP less and ceruloplasmin more abundantly than did the other two cell lines (Table 2). The differences among the cell lines are probably due to the degree to which they maintain liver-specific gene expression, which may reflect the expression of liver-specific transcription factors and hepatic nuclear factors (HNFs), or to genetic differences at the germ-line level or somatic mutations.

When the profiles of secretory proteins were compared between the monolayer and the RFB cultures for each cell line, FLC5 appeared to differ from the two other cell lines. Ratios of albumin (+

hemopexin), transferrin, transthyretin, SRBP, and ceruloplasmin were higher abundant in RFB culture than in monolayer culture for FLC5, but this tendency was not observed for FLC4 or FLC7.

There were large discrepancies in the ratio of components between the proteins secreted by the FLC cell lines and those found in human serum. Albumin accounts for only 9% to 30% of total secreted proteins from FLC cells under either culture condition (Fig. 2) but normally accounts for more than 50% of total serum protein in healthy persons[10]. In contrast, α1-antitrypsin/α1-antichymotrypsin and transferrin were both major components in protein secreted by FLC cells (Fig. 2) but are minor components in normal serum, with α1-antitrypsin/α1-antichymotrypsin being approximately 0.02% of total protein and transferrin being less than 0.1%[10]. The discrepancies are probably attributable to the difference in the stability or clearance of proteins between culture conditions and in the body.

We identified at least 14 protein spots on the gels, by means of MALDI-TOF mass spectrometry and comparison with a public database, but we could not identify the majority of protein spots. Most of these unidentified proteins were minor components, accounting for 40% to 60% of all secretory proteins (Fig. 2). Identification of the entire set of protein spots will contribute much to characterization and to proper cell selection. It should be noted, however, that selection of an appropriate cell line for clinical use of the bioartificial liver would also be affected by cellular functions other than protein secretion, particularly by detoxification.

In conclusion these results indicate that the FLC cell lines secret several physiologically important liver-specific proteins under both monolayer culture and RFB culture conditions. The proportions of each protein, however, varied between the cell lines. The results provide useful basic data for selection of appropriate cell lines in the clinical and experimental applications of the bioartificial liver.

Acknowledgements: We thank Ms. Hiroko Takizawa and Ms. Haruka Maehashi for technical assistance. This study was supported in part by grants-in-aid from the Promotion and Mutual Aid Corporation for Private School of Japan, University Start-Ups Creation Support System, and the Japan Health Sciences Foundation (Research on Health Sciences focusing on Drug Innovation, KH71068) and a grant for graduate students from The Jikei University School of Medicine.

REFERENCES

1. Nagamori S, Hasumura S, Matsuura T, Aizaki H, Kawada M. Developments in bioartificial liver research: concept, function and application. J Gastroenterol 2000; 35: 493-503.
2. van de Kerkhove MP, Hoekstra R, Chamuleau RA, van Gulik TM. Clinical application of bioartificial liver support systems. Ann Surg 2004; 240: 216-30.
3. Kawada M, Nagamori S, Aizaki H, Fukaya K, Niiya M, Matsuura T, et al. Massive culture of human liver cells in a newly developed radial flow bioreactor system: ultrafine structure of functionally enhanced hepatocarcinoma cell lines. In Vitro Cell Dev Biol 1998; 34: 109-15.
4. Fujise K, Nagamori S, Hasumura S, Homma S, Sujino H, Matsuura T, et al. Integration of hepatitis B virus DNA into cells of six established human hepatocellular carcinoma cell lines. Hepato Gastroenterol 1990; 37: 457-60.
5. Iwahori T, Matsuura T, Maehashi H, Sugo K, Saito M, Hosokawa M, et al. CYP3A4 inducible model for in vitro analysis of human drug metabolism using a bioartificial liver. Hepatology 2003; 37: 665-73.
6. Aizaki H, Nagamori S, Matsuda M, Kawakami H, Hashimoto O, Ishiko H, et al. Production and release of infectious hepatitis C virus from human liver cell cultures in the three-dimensional radial-flow bioreactor. Virology 2003; 314: 16-25.
7. O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 1975; 250: 4007-21.
8. Hirano H. Varietal differences of leaf protein profiles in mulberry. Phytochemistry 1982; 21: 1515-28.
9. Hoogland C, Mostaguir K, Sanchez J-C, Hochstrasser DF, Appel RD. SWISS-2DPAGE, ten years later. Proteomics 2004; 4: 2352-6.
10. Ganong WF. Review of medical physiology. 22nd ed. New York: McGraw-Hill, 2005: 540-1.