Formation of porcine hepatocyte spherical multicellular aggregates (spheroids) and analysis of drug metabolic functions

Kohji Nakazawa¹, Hiroshi Mizumoto¹, Mitsuru Kaneko¹, Hiroyuki Ijima¹, Tomonobu Gion², Mitsuo Shimada², Ken Shirabe², Kenji Takenaka², Keizo Sugimachi² & Kazumori Funatsu^{1,*}

¹ Department of Chemical Systems and Engineering, Graduate School of Engineering, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan (* Author for all correspondence.)

² Department of Surgery II, Faculty of Medicine, Kyushu University, 3-1-1 Maldashi, Higashi-ku, Fukuoka 812-8582, Japan

Received 18 August 1998; accepted 25 December 1998

Key words: acetaminophen metabolism, lidocaine metabolism, monolayer culture, polyurethane foam, porcine hepatocytes, spheroid culture

Abstract

Porcine hepatocytes are used in the hybrid artificial liver support system that we are developing because of their high level of liver functions *in vitro* and because human hepatocytes can not be used in Japan for ethical reasons. Spherical multicellular aggregates or spheroids have been found to be effective *in vitro* for long-term maintenance of liver functions. Therefore, we formed spherical multicellular aggregates (spheroids) of primary porcine hepatocytes using a polyurethane foam (PUF) as a culture substratum and analyzed their drug metabolic functions *in vitro*. Primary porcine hepatocytes inoculated into the pores of a flat PUF plate (25 × 25 × 1 mm), spontaneously formed spheroids within the range of 100 to 150 µm in diameter 24 to 36 h after inoculation. The formed spheroids were attached to the bottom surface of the PUF pores, and their morphology and viability were maintained for more than 12 days. The P-450 activity in the spheroids of porcine hepatocytes was demonstrated by detecting production of monoethylglycinexylidide from lidocaine. In addition, the conjugation enzyme activity was demonstrated by detecting glucuronidation and sulfation of acetaminophen. These activities were maintained for 12 days at a level twice as high as in the monolayer culture. This result shows that the porcine hepatocyte spheroids formed by using PUF can maintain the drug metabolic functions important in a hybrid artificial liver device. Consequently, culturing porcine hepatocyte spheroids using PUF seems to be promising for development of a hybrid artificial liver.

Abbreviations: MEGX, monoethylglycinexylidide; PUF, polyurethane foam

Introduction

A hybrid artificial liver support system composed of a hepatocyte culture module and an extracorporeal circulation system has been developed as a potential bridge to liver transplantation and an effective treatment for fulminant hepatic failure in humans. Two important considerations of the hybrid artificial liver are the level and length of time the hepatocytes can express their liver-specific functions. Many methods for maintaining high level functions of cul-

tured hepatocytes have been investigated, including the use of extracellular matrix components (Dunn et al., 1992; Berthiaume et al., 1996) and co-culturing with liver-derived nonparenchymal cells (Guguen et al., 1983). Recently, much attention has been focused on spherical multicellular aggregates (spheroids) that have assembled from dispersed hepatocytes. Many researchers have reported various techniques to form hepatocyte spheroids. Several of these methods use a dish coated with an extracellular matrix protein such as proteoglycan (Koide et al., 1989, 1990), poly-(2-

hydroxyethyl methacrylate) (Landry et al., 1985; Tong et al., 1992), poly-N-isopropyl acrylamide (Takezawa et al., 1992) or Eudragit S100 (Kamihira et al., 1997), as a culture substratum. In addition, agitated tank systems have also been implemented (Sakai et al., 1992). Hepatocytes in spheroids can maintain higher levels of liver-specific functions than dispersed hepatocyte culture, presumably due to cell-cell interactions.

We reported that primary rat hepatocytes spontaneously formed spheroids 100 to 150 μm in diameter in the pores of polyurethane foam (PUF). The PUF has a sponge-like macroporous structure with each pore made up of smooth thin films (average pore size: about 300 μm). Rat hepatocyte spheroids can maintain liver specific functions, such as albumin secretion and urea synthesis, for at least one month (Matsushita et al., 1991; Ijima et al., 1992). Furthermore, the hepatocyte spheroids are immobilized at the bottom surface of PUF pores, a very advantageous culture methods. A high cell density of more than 1.0×10^7 cells cm^{-3} -PUF can be achieved. Therefore we have been developing an artificial liver employing a PUF packed-bed module housing rat hepatocyte spheroids. The effectiveness of this device was demonstrated by testing it on rats with liver failure (Matsushita et al., 1995).

Primary porcine hepatocytes are attractive as a biological component of a hybrid artificial liver for human application because pigs are thought to have a physiology similar to that of humans. Some studies have used devices containing porcine hepatocytes as a bridge for liver transplantation in patients (Chen et al., 1996; Gerlach 1997).

This paper reports the results of the formation of porcine hepatocyte spheroids using PUF as a culture substratum. Also, drug metabolic functions are demonstrated by measuring the phase I activity of lidocaine and the phase II activity of acetaminophen. Efficient formation of porcine hepatocyte spheroids and high drug metabolism are necessary to justify their use in a human artificial liver.

Materials and methods

Materials

Unless otherwise noted, all chemical and cell culture reagents were purchased from Sigma Chemical Co., St. Louis, U.S.A.. Lidocaine and acetaminophen as marker drugs were purchased from Nakarai Tesque,

Japan. A polystyrene dish of 35 mm in diameter and a Type I-collagen-coated dish of 35 mm in diameter manufactured by Iwaki, Japan, were used to culture PUF/spheroids and monolayers, respectively.

The culture medium was a hormone-defined medium (HDM) comprised of williams'E medium supplemented with 7.23 mg l^{-1} insulin, 25 $\mu\text{g l}^{-1}$ epidermal growth factor (EGF) (Funakoshi, Japan), 0.1 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 3 nM H_2SeO_3 , 50 pM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 50 $\mu\text{g l}^{-1}$ linoleic acid, 20 $\mu\text{g l}^{-1}$ liver growth factor, 1 μM dexamethasone, 6.25 mg l^{-1} transferrin, 500 mg l^{-1} albumin, 4 $\mu\text{g l}^{-1}$ glucagon, 50 mg l^{-1} gentamycin and 50 mg l^{-1} chloramphenicol (Lazar et al., 1995).

The PUF was kindly donated by INOAC Co. Ltd., Japan. A flat PUF plate ($25 \times 25 \times 1$ mm) was cut from a large PUF block, washed several times in distilled water, and then autoclaved for sterilization. Before use, it was washed again with williams'E medium and soaked in HDM before inoculation.

Preparation of porcine hepatocytes

Porcine hepatocytes were isolated from domestic pigs 5 to 6 weeks old weighting approximately 10 kg (Kyudo, Japan) by liver perfusion (Seglen, 1976; Gerlach et al., 1994). Briefly, a liver obtained from a pig was perfused with 0.1% collagenase type IV at a flow rate of 200 to 800 ml min^{-1} for about 20 min, and the hepatocytes were dispersed from the liver. The dispersed hepatocytes were filtered through a 106 μm stainless steel mesh and were washed with fresh williams'E medium. The hepatocytes were resuspended in fresh HDM and were inoculated into the PUF. The average number of cells in three experiments was $(1.5 \pm 0.3) \times 10^{10}$ cells, and the average cell viability was more than 85% measured by trypan blue exclusion.

Monolayer culture and PUF/spheroid culture of porcine hepatocytes

To prepare the monolayer culture, a porcine hepatocyte suspension at cell density 2.5×10^5 cells ml^{-1} in HDM was inoculated into a Type I-collagen-coated dish at 2 ml per dish. The medium was changed 4 h after inoculation and thereafter at 2-day intervals.

For PUF/spheroid culture, 2 ml of suspension at cell density 1.0×10^6 cells ml^{-1} in HDM was inoculated into a flat PUF plate ($25 \times 25 \times 1$ mm) in a polystyrene dish. Four hours after inoculation, the PUF plates were transferred to another dish containing

2 ml fresh medium to remove the hepatocytes that had not attached. By using this procedure, about 25% of the inoculated hepatocytes were immobilized in the pores of PUF plate. Thereafter, cultured HDM was replaced with the equivalent fresh medium at 2-day intervals.

Both monolayer and PUF/spheroid cultures were cultivated in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Measurement of viable cells

To evaluate a time course of viable cells in both cultures, we used the MTT method (Mosmann, 1983). Briefly, 0.5 ml of MTT solution, which was dissolved with PBS buffer at 5 mg ml⁻¹, was added to each dish containing cultured hepatocytes and 2 ml of medium. After 12 h of incubation, MTT formazan converted from MTT by mitochondria in the hepatocytes was extracted by 3 ml of 0.04 N HCl-isopropanol. The difference in absorbance between 560 and 690 nm of the extracted samples was measured by a spectrophotometer (HITACHI U-2000, Japan).

Calibration curves of viable cells and absorbance were constructed by measuring isolated hepatocytes in the range from 1.0×10^4 to 1.0×10^6 viable cells.

Lidocaine and acetaminophen metabolism

To evaluate phase I (P-450) and phase II (conjugation enzyme) activities of drug metabolism, the HDM in both cultures was removed and replaced with 2 ml of fresh HDM containing 58.5 µg ml⁻¹ (0.25 mM) lidocaine or 151 µg ml⁻¹ (1.0 mM) acetaminophen. Drug metabolic reactions were carried out every 4 days on a rotary shaker at 45 rpm for 24 h in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Lidocaine and its metabolite, monoethylglycinexylidide (MEGX), were extracted from the spent medium and the cells using 2 ml toluene and then were analyzed using gas chromatography/mass spectrometry (GC/MS, Hewlett Packard 5890, U.S.A.) according to the procedure described previously (Nakazawa et al., 1997). Acetaminophen and its glucuronidated and sulfated metabolites were extracted from the spent medium and the cells using 2 ml ethanol and then were analyzed using high performance liquid chromatography (HPLC, Hitachi L-6000, Japan) according to the procedure of Peter (1978).

The lidocaine and acetaminophen metabolic rates were normalized by viable cell numbers obtained by

the MTT method. The results are the mean $\pm$ standard deviation (SD) of three experiments.

In preliminary experiments, it was confirmed that lidocaine and acetaminophen were not converted to metabolites in the cultures without hepatocytes and that these marker drugs could be recovered completely from HDM by the extraction procedures described above.

Results

Morphology of porcine hepatocytes

Porcine hepatocytes inoculated onto the Type I-collagen-coated dish spread within 4 h, and then formed a monolayer (Figure 1a). The monolayer morphology was maintained through 12 days of culture.

However, the porcine hepatocytes inoculated into the flat PUF plate attached to the internal surface of PUF pores, and most of inoculated hepatocytes began to aggregate within 4 h (Figure 1b). The hepatocytes gathered spontaneously and made loose cell aggregates in the PUF pores within 12 to 24 h (Figure 1c). Furthermore, the cells rearranged and compacted, and multicellular spheroids 100 to 150 µm in diameter formed in the PUF pores after 24 to 36 h of inoculation (Figure 1d).

Most of the hepatocytes attached to the bottom surface of PUF pores formed spheroids. A stable spheroid morphology was maintained for at least 12 days.

Time courses of viable cells in monolayer and PUF/spheroid culture

Figure 2 shows the time courses of viable cells in both cultures. By using the MTT method, the viable cell numbers in the monolayer culture and the PUF/spheroid culture were maintained at about 2.5×10^5 cells/dish and 3.3×10^5 cells/dish, respectively, throughout 12 days of culture.

Lidocaine metabolism

The cytochrome P-450 activities of porcine hepatocytes in both cultures were evaluated by lidocaine metabolism. The biotransformation of lidocaine to MEGX by N-deethylation was identified by the mass fragment pattern of GC/MS.

Figure 3 shows the time course of lidocaine disappearance rates in both cultures. In the monolayer culture, the initial lidocaine disappearance rate was

Figure 1. Photomicrographs of primary porcine hepatocytes. (A) Porcine hepatocytes on Type I-collagen-coated dish at 24 h of culture. Hepatocytes attached and spread on the dish, and then formed a monolayer. (B) Hepatocytes in PUF pores at 5 h of culture. Hepatocytes began to aggregate. (C) Hepatocytes in PUF pores at 15 h of culture. They gathered spontaneously and made loose cell aggregates. (D) Hepatocytes in PUF pores at 28 h of culture. After they rearranged and compacted, spheroids were formed. Bar: 200 μm .

about $38 \mu\text{g}/10^5$ viable cells/day and then gradually decreased. After 8 days of culture, the rate decreased to about $8 \mu\text{g}/10^5$ viable cells/day.

In the PUF/spheroid culture, the lidocaine concentration decreased from $250 \mu\text{M}$ to below $5 \mu\text{M}$ within 24 h at the first and fourth days of culture. The initial lidocaine disappearance rate, about $32 \mu\text{g}/10^5$ viable cells/day, was slightly low compared with monolayer hepatocytes, but it was maintained until 4 days of culture. At that time the rate gradually decreased and was about $18 \mu\text{g}/10^5$ viable cells/day at 12 days of culture. However, the activity in the spheroid culture was maintained at about twice the level of that in the monolayer culture for at least 12 days.

MEGX was formed in both cultures, but its quantity was very low (less than $1 \mu\text{g}$). Furthermore, the MEGX appearance rates in both cultures were not

markedly different between the cultures through 12 days.

Acetaminophen metabolism

The glucuronide and sulfate conjugational activities of porcine hepatocytes in both cultures were evaluated by acetaminophen metabolism. Glucuronidation and sulfation of acetaminophen were identified by the retention time of HPLC analysis as in the study by Peter (1978).

Figure 4 shows the time course of acetaminophen disappearance rates in both cultures. In the monolayer culture, the initial acetaminophen disappearance rate of porcine hepatocytes was about $4.7 \mu\text{g}/10^5$ viable cells/day and then dramatically decreased. After 4 days of culture, the rate was about $0.5 \mu\text{g}/10^5$ viable cells/day.

Figure 2. Time courses of viable cell numbers in monolayer and spheroid cultures. The data represent the mean $\pm$ standard deviation (SD) of 3 experiments. —●—: Monolayer culture. —○—: PUF/spheroid culture. The viable cell numbers in both culture were constant throughout the 12 days of culture.

However, in the PUF/spheroid culture, the initial acetaminophen disappearance rate was about $5.7 \mu\text{g}/10^5$ viable cells/day, and the activity was remained constant for 4 days of culture. After this, although the rate gradually decreased, it was maintained at about $2 \mu\text{g}/10^5$ viable cells/day after 8 days of culture. Similar to the result of lidocaine metabolism, the acetaminophen disappearance activity in the spheroid culture was also maintained at about twice as high as that in the monolayer culture for at least 12 days.

An appearance ratio of acetaminophen glucuronide and sulfate (AG:AS) in both cultures was about 1:9 through 12 days. The sulfate conjugational activity of acetaminophen in porcine hepatocytes was higher than the glucuronide conjugational activity.

Discussion

Primary porcine hepatocytes are significant biological components in hybrid artificial liver devices developed

for clinical applications (Komlot et al., 1995; Naruse et al., 1996; Hu et al., 1997). Therefore, because porcine hepatocyte spheroids show improved cell viability and enhanced liver specific functions, they have attracted attention for future clinical uses.

In this paper, we demonstrated spheroid formation of porcine hepatocytes using PUF as a culture substratum. Porcine hepatocyte spheroid formation using an agitated tank has also been reported (Sakai et al., 1996; Lazar et al., 1995). Although the mechanism of spheroid formation in the pores of PUF is not yet clear, the mechanisms of spheroid formation using PUF or an agitated tank may be different. Spheroid formation using PUF is spontaneous, but spheroid formation in an agitated tank requires stirring. We confirmed a proteoglycan accumulation on the internal surface of PUF (data not shown). Therefore, we speculate that the cellular affinity for PUF and extracellular matrices (proteoglycan, etc.) may affect spheroid formation.

Maintenance of viable cells requires good mass transfer to supply oxygen and nutrients and remove metabolic wastes. Therefore, the diameter of the

Figure 3. Time courses of lidocaine disappearance rates of porcine hepatocytes in monolayer and PUF/spheroid cultures. The data represent the mean $\pm$ SD of 3 experiments. —●—: Monolayer culture. —○—: PUF/spheroid culture. The activity in the monolayer culture gradually decreases. Although the activity in the spheroid culture also gradually decreases, the level is maintained at about twice as high as that in the monolayer culture for at least 12 days.

spheroid is important because the mass transfer in the center of a spheroid is limited by diffusion. In the spheroid culture using PUF, the diameter is controlled by the inoculation cell density (Matsushita et al., 1992). In this study, the diameter of the spheroids in the pores of PUF was kept within 100 to 150 μm , and cell viability remained constant for at least 12 days. Spheroids as large as 150 μm in diameter are not necrotic when formed in PUF. Furthermore, the dispersed porcine hepatocytes rapidly formed spheroids in PUF pores within about 24 h, indicating that a hybrid artificial liver using PUF/spheroid culture can be prepared in about 24 h for use.

In the recent studies of hepatocyte culture, lidocaine metabolism has become recognized as a phase I (oxidation, reduction, etc.) activity of drug metabolism (Nyberg et al., 1977; Sielaff et al., 1995). Lidocaine is converted by multiple cytochrome P-450 isoenzymes (Bargetzi et al., 1989) in the endoplasmic reticulum of hepatocytes. The appearance of the lidocaine metabolite, MEGX, also indicated the role

of P-450 activity (Oellerich et al., 1987). Primary hepatocytes in a monolayer culture lose P-450 activity within a few days of culture (Bader et al., 1992). Loss of monolayer activity was also shown in this study (Figure 3). In contrast, the P-450 activity in the PUF/spheroid culture was twice as high as that in the monolayer culture for at least 12 days, indicating that a PUF/spheroid culture can maintain stable P-450 activity. Presumably the three-dimensional structure of spheroid may be able to keep the functions of the endoplasmic reticulum in hepatocytes. However, the MEGX appearance was very low in this study, probably because the MEGX was rapidly converted to other compounds and/or the MEGX was insufficiently recovered from the sample in our extraction procedure.

Lazar et al. (1995) reported that the lidocaine disappearance rate in porcine hepatocyte spheroids was about 2.8 $\mu\text{g}/10^5$ cells/day, but the rate in our study was about 25 $\mu\text{g}/10^5$ cells/day, which was about 10 times higher than in Lazar's study. This difference may be due to the difference in the initial lidocaine

Figure 4. Time courses of acetaminophen disappearance rates of porcine hepatocytes in monolayer and PUF/spheroid cultures. The data represent the mean $\pm$ SD of 3 experiments. —●—: Monolayer culture. —○—: PUF/spheroid culture. The activity in the monolayer culture rapidly decreases, but the activity in spheroid culture is maintained at about twice as high as that in the monolayer culture for at least 12 days.

concentration ($58.5 \mu\text{g ml}^{-1}$ in our study compared to $12.0 \mu\text{g ml}^{-1}$ in Lazar's study) since the activity of drug metabolism depends on drug concentration. Although evaluating the same lidocaine concentrations is necessary to clearly compare the spheroid cultures, this result suggests that the lidocaine metabolic activity of the PUF/spheroid culture is similar to spheroids formed in an agitated tank.

Acetaminophen was used to evaluate phase II (conjugation) drug metabolism. The biotransformation of acetaminophen by conjugation to glucuronide and sulfate required conjugational enzymes, such as glucuronosyltransferase and sulfotransferase in hepatocytes (Studenberg et al., 1991). In this study, we demonstrated that the acetaminophen disappearance activity in the PUF/spheroid culture was maintained longer than that in the monolayer culture for at least 12 days, suggesting that spheroid morphology can maintain a stable conjugational enzyme activity. Furthermore, because the appearance ratios of glucuronide and sulfate in both cultures were constant during the

culture period, we consider that the drug metabolic pathway in porcine hepatocytes does not change in both cultures.

In summary, primary porcine hepatocytes formed spheroids in the pores of PUF as a culture substratum, and their drug phase I and phase II metabolic activities were maintained at about twice as high as a hepatocyte monolayer for at least 12 days. Although other functions in hepatocyte spheroids must be evaluated, PUF/porcine hepatocyte spheroid culture seems to be promising to develop a hybrid artificial liver for a short-term use.

Acknowledgements

This study was supported by Grant-in-Aids for Developmental Scientific Research (A)(2): 10305062 and (C)(2): 09650882 from the Ministry of Education, Science, Sports and Culture, and by Kyushu University Interdisciplinary Programs in Education and Projects

in Research Development of B Type, and in part by the Inamori Foundation.

References

- Bader A, Christians U, Boker K, Sattler M, Sewing K and Pichmayr R (1992) In vitro imitation of the in vivo three-dimensional microenvironment enables primary hepatocytes to maintain stable metabolic functions. *Cell Transplantation* 1: 162.
- Bargetzi MJ, Aoyama T, Gonzalez FJ and Meyer UA (1989) Lidocaine metabolism in human liver microsomes by cytochrome P450III_{A4}. *Clin Pharmacol Ther* 46: 521–527.
- Berthiaume F, Moghe PV, Toner M and Yarmush M (1996) Effect of extracellular matrix topology on cell structure, function, and physiological responsiveness: hepatocytes cultured in a sandwich configuration. *FASEB J* 10: 1471–1484.
- Chen SC, Hewitt WR, Watanabe FD, Eguchi S, Kahaku E, Middleton Y, Rozga J and Demetriou AA (1996) Clinical experience with a porcine hepatocyte-based liver support system. *Int J Artif Organs* 19: 664–669.
- Dunn J, Tompkins R and Yarmush M (1992) Hepatocytes in collagen sandwich: evidence for transcriptional and translational regulation. *J Cell Biol* 116: 1043–1053.
- Gerlach JC (1997) Bioreactor for a hybrid liver support. In: Carondo MJT et al. (eds) *Animal Cell Technology* (pp. 543–555), Kluwer Academic Publishers, The Netherlands.
- Gerlach JC, Brombacher J, Kloppel K, Schnoy N and Neuhaus P (1994) Comparison of four methods for mass hepatocyte isolation from pig and human liver. *Transplantation* 57: 1318–1322.
- Guguen GC, Clement B, Baffet G, Beaumont C, Morel CE and Guilouzo A (1983) Maintenance and reversibility of active albumin secretion by adult rat hepatocytes co-cultured with another liver epithelial cell type. *Exp Cell Res* 143: 47–54.
- Hu WS, Friend JR, Wu FJ, Sielaff T, Peshwa MV, Lazar A, Nyberg SL, Rummel RP and Cerra FB (1997) Development of a bioartificial liver employing xenogeneic hepatocytes. *Cytotechnology* 23: 29–38.
- Ijima H, Taniguchi Y, Matsushita T and Funatsu K (1992) Application of three dimensional culture of adult rat hepatocytes in PUF pores for artificial liver support system. In: Murakami H et al. (eds) *Animal Cell Technology: Basic and Applied Aspects* (pp. 81–86), Kluwer Academic Publishers, The Netherlands.
- Kamihira M, Yamada K, Hamamoto R and Iijima S (1997) Spheroid formation of hepatocytes using synthetic polymer. In: Prokop A et al. (eds) *Annals of the New York Academy of Sciences*. vol. 831 (pp. 398–407), The New York Academy of Sciences, New York.
- Koide N, Sakaguchi K, Koide Y, Asano K, Kawaguchi M, Matsushita H, Takenami T, Shinji T, Mori M and Tsuji (1990) Formation of multicellular spheroids composed of adult rat hepatocytes in dishes with positively charged surfaces and under other nonadherent environments. *Exp Cell Res* 186: 227–235.
- Koide N, Shinji T, Tanabe T, Asano K, Kawaguchi M, Sakaguchi K, Koide Y, Matsushita H, Mori M and Tsuji (1989) Continued high albumin production by multicellular spheroids of adult rat hepatocytes formed in the presence of liver-derived proteoglycans. *Biochem Biophys Res Comm* 161: 385–391.
- Komlot A, Rozga J, Watanabe FD and Demetriou AA (1995) Artificial liver support systems. *Biotechnol Bioeng* 50: 382–391.
- Landry J, Bernier D, Ouellet C, Goyette R and Marceau N (1985) Spheroidal aggregate culture of rat liver cells: Histotypic reorganization, biomatrix deposition, and maintenance of functional activities. *J Cell Biol* 101: 914–923.
- Lazar A, Mann HJ, Rummel RP, Shatford RA, Cerra FB and Hu WS (1995) Extended liver-specific functions of porcine hepatocyte spheroids entrapped in collagen gel. *In Vitro Cell Dev Biol* 31: 340–346.
- Matsushita T, Ijima H, Wada S and Funatsu K (1995) Estimation of the performance of a PUF/spheroid packed-bed type artificial liver by using an extracorporeal circulation with hepatic failure rats. *Jpn J Artif Organs* 24: 815–820.
- Matsushita T, Ijima H and Funatsu K (1992) Development of a hybrid type artificial liver utilizing three dimensional culture of adult hepatocytes. *Jpn J Artif Organs* 21: 1050–1054.
- Matsushita T, Ijima H, Koide N and Funatsu K (1991) High albumin production by multicellular spheroid of adult rat hepatocytes formed in the pores of polyurethane foam. *Appl Microbiol Biotechnol* 36: 324–326.
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55–63.
- Nakazawa K, Matsushita T and Funatsu K (1997) Prolonged lidocaine metabolizing activity of primary hepatocytes with spheroid culture using polyurethane foam as a culture substratum. *Cytotechnology* 24: 235–242.
- Naruse K, Sakai Y, Nagashima I, Jiang GX, Suzuki M and Muto T (1996) Comparisons of porcine hepatocyte spheroids and single hepatocytes in the non-woven fabric bioartificial liver module. *Int J Artif Organs* 19: 605–609.
- Nyberg G, Karlen B, Heldlund I, Grundin R and Bahr C (1977) Extraction and metabolism of lidocaine in rat liver. *Acta Pharmacol Toxicol* 40: 337–346.
- Oellerich M, Raude E, Burdelski M, Schulz M, Schmidt FW, Ringe B, Lamesch P, Pichlmayr R, Raith H, Scheruhn M, Wrenger M and Wittekind C (1987) Monoethylglycinexylidide formation kinetics: a novel approach to assessment of liver function. *J Clin Chem Clin Biochem* 25: 845–853.
- Peter M (1978) Paracetamol metabolism and toxicity in isolated hepatocytes from rat and mouse. *Biochem Pharmacol* 27: 2859–2863.
- Sakai Y, Naruse K, Nagashima I, Muto T and Suzuki M (1996) Large-scale preparation and function of porcine hepatocyte spheroids. *Int J Artif Organs* 19: 294–301.
- Sakai Y, Furukawa K and Suzuki M (1992) Immobilization and long-term albumin secretion of hepatocyte spheroids rapidly formed by rotational tissue culture methods. *Biotech Techniques* 6: 527–532.
- Seglen PO (1976) Preparation of isolated rat liver cells. In: Prescott DM (ed) *Methods Cell Biol.* vol. 113 (pp. 29–83), Academic press, New York.
- Sielaff TD, Hu MY, Rao S, Groehler K, Olson D, Mann HJ, Rummel RP, Shatford RAD, Amiot B, Hu WS and Cerra FB (1995) A technique for porcine hepatocyte harvest and description of differentiated metabolic functions in static culture. *Transplantation* 59: 1459–1463.
- Studenberg SD and Brouwer KLR (1991) Phenacetin and acetaminophen metabolism in the isolated perfused rat liver. *Drug Metab Dispos* 19: 423–429.
- Takezawa T, Yamazaki M, Mori Y, Yonaha T and Yoshizato K (1992) Morphological and immuno-cytochemical characterization of a hetero-spheroid composed of fibroblasts and hepatocytes. *J Cell Science* 101: 495–501.
- Tong JG, Lagaussie PD, Furlan V, Cresteil T, Bernard O and Alvarez F (1992) Long-term culture of adult rat hepatocyte spheroids. *Exp Cell Res* 200: 326–332.