Prolonged lidocaine metabolizing activity of primary hepatocytes with spheroid culture using polyurethane foam as a culture substratum

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Abstract

Primary rat hepatocytes formed spheroids in the pores of polyurethane foam (PUF) used as a culture substratum. The hepatocytes in monolayer and spheroid stationary culture converted lidocaine to monoethylglycinexylidide (MEGX) which was N-deethylation of lidocaine. The metabolic activity of the hepatocytes/spheroid stationary culture system was 1.5~2.0-fold higher than that of monolayer culture for 10 days. The activity of albumin production and cell survival of hepatocytes in monolayer and spheroid cultures decrease due to lidocaine treatment dependent on the lidocaine concentration, but the activity and cell survival in PUF/spheroid stationary culture were maintained at a higher level than that in monolayer culture under the lidocaine treatment. We developed a device for an *in vitro* liver model, drug metabolism simulator (DMS), using a PUF/spheroid packed-bed module including $4.00 \pm 0.68 \times 10^7$ hepatocytes and analyzed pharmacokinetics of lidocaine in a one-compartment model. Lidocaine clearance and extraction ratio of hepatocytes in the DMS corresponded to 1.354 ± 0.318 ml/min/g-liver and 0.677 ± 0.0159 /g-liver, respectively (N=4). These values were comparable with *in vivo* values, 1.930 ml/min g-liver and 0.965/g-liver reported by Nyberg (1977). Consequently, PUF/spheroid culture maintained high lidocaine metabolizing activity over a long term and seems to provide a promising culture system as a drug metabolism simulator which will be used for drug screening, cytotoxicity tests and prediction of pharmacokinetics.

Abbreviations: DMS – drug metabolism simulator; MEGX – monoethylglycinexylidide; PUF – polyurethane foam

Introduction

In vitro models using cultured cells are increasingly used to investigate toxic effects and metabolism of new chemicals (Goldberg et al., 1989). Hepatocytes especially are frequently the target cells of chemically mediated toxicity because the liver is an important organ in biotransformation of xenobiotics.

Although *in vitro* experiments using hepatoma cell lines are easier and more reproducible, these cells in culture generally show more or less abnormal characteristics in comparison with primary hepatocytes (Doostdar et al., 1990).

Therefore, primary hepatocytes are often used for these experiments, but in the usual monolayer culture using culture dishes, these differentiated functions

rapidly decrease in a week or so *in vitro* (Bader et al., 1992). For that, hepatocyte/monolayer culture is only used in a short term study of drug evaluation, and seems to be difficult to use in a long term toxicity test of new drugs. Therefore, development of culture technology of primary hepatocytes which make it possible to maintain these functions stably *in vitro*, is very important. Actually, recent developments in cell culture technology have sustained long-term survival and maintained hepatocyte differentiated functions including cultivation of hepatocytes as spheroids (Koide et al., 1989) and collagen gel immobilization (Dunn et al., 1989). By these technologies, the effective utilization of valuable cells such as human hepatocytes will become possible.

We have already reported that by three-dimensional culture of rat hepatocytes (spheroid culture) using polyurethane foam (PUF) as a culture substratum, hepatocyte specific functions such as albumin production and urea synthesis can be maintained for more than 2 weeks (Matsushita et al., 1991). Furthermore, we indicated that PUF/spheroid culture was applicable to the development of a hybrid artificial liver support system (Ijima et al., 1994).

In this study, we investigated the lidocaine metabolizing activity of primary hepatocytes/spheroid culture using PUF for the development of a simulation device of drug metabolism of liver *in vitro*. Lidocaine was used as a model drug of phase I (oxidation/reduction) metabolism of hepatocytes, which indicated P450 activity. Pharmacokinetic analysis of lidocaine metabolism using PUF/spheroid packed-bed was also investigated to quantify the activity of drug metabolism simulators including hepatocyte/spheroid.

Materials and methods

Materials

Lidocaine [2-Diethylamino-N-(2,6-dimethylphenyl)-acetamide] as a model drug of drug metabolism of hepatocytes and *p*-diethoxybenzene for internal standard (IS) of quantitative analysis of GC/MS were purchased from NAKARAI TESQUE (Japan) and WAKO CHEMICALS (Japan), respectively. MTT for measurement of viable cell numbers were from Sigma (MO, USA). Petri dishes (diameter: 35 mm) and typeI-collagen coated dishes (diameter: 35 mm) were from IWAKI (Japan).

Hormone-defined medium (HDM) was used in this experiment. HDM comprised Williams'E medium supplemented with $10 \mu\text{g ml}^{-1}$ insulin (Sigma Co., USA), $0.1 \text{ mM CuSO}_4 \cdot 5\text{H}_2\text{O}$, $3 \text{ ng ml}^{-1} \text{ H}_2\text{SeO}_3$, $50 \text{ pM ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 50 ng ml^{-1} (FUNAKOSHI), 50 mg ml^{-1} linoleic acid (Sigma Co., USA), $58.8 \mu\text{g ml}^{-1}$ penicillin, $100 \mu\text{g ml}^{-1}$ streptomycin and $1 \mu\text{g ml}^{-1}$ amphotericin B.

PUF was kindly donated by INOAC Co., Ltd. (Nagoya, Japan). PUF has a sponge-like macroporous structure with each pore made up of smooth thin films and thick skeletons as reported in a previous paper (Matsushita et al., 1991). The average pore diameter of PUF is about $500 \mu\text{m}$. A block of PUF was cut into flat plates ($1 \times 25 \times 25 \text{ mm}$) for stationary culture and cylindrical shape (diameter; 12 mm, length; 50

mm) for packed-bed culture. Before use, the PUF substrates were submerged and washed in distilled water to remove any chemical contaminants, and autoclaved in order to sterilize them. Then, the PUF substrates were washed again with Williams'E medium and soaked in HDM prior to cell inoculation.

Hepatocytes preparation and culture

Hepatocytes were isolated from adult Wistar rats (7 weeks, 180~230 g) by the liver perfusion method (Seglen, 1976) with 0.05% collagenase (WAKO CHEMICALS). Cell viability was determined by the trypan blue exclusion method. In this experiment, the viability of isolated hepatocytes was more than 80%.

The isolated hepatocytes were inoculated for monolayer culture as a density of 5.0×10^5 viable cells per typeI-collagen coated dishes (diameter: 35 mm), in 2 ml of medium under a humidified atmosphere of 5% CO_2 and 95% air at 37°C . In the case of monolayer culture, the medium was changed after 4 h of culture and thereafter at 2-day intervals.

For PUF/spheroid stationary culture, the isolated hepatocytes were inoculated at a density of 3.0×10^6 viable cells per petri dish which contained a piece of PUF plate. After 4 h of culture, the inoculated PUF plates were transferred to another petri dish containing 2 ml fresh medium and thereafter the medium was changed at 2-day intervals, and cultured under a humidified atmosphere of 5% CO_2 and 95% air at 37°C .

Drug metabolism simulator (DMS) using PUF/spheroid packed-bed

We developed a device (drug metabolism simulator; DMS) as an *in vitro* liver model for continuous measurement of drug metabolism using PUF/spheroid packed-bed as shown in Fig. 1. DMS was composed of a cell immobilized module, reserve bottle of medium, gas exchange apparatus etc. The isolated hepatocytes were inoculated by a centrifugal method at a density of 7.0×10^7 cells per multicapillary (MC)-PUF module (diameter, 12 mm, length; 50 mm, volume; 4.4 cm^3) (Ijima et al., 1994). The total volume of cultured medium in this device was 50 ml. The flow rate in the lines was 4.5 ml/min. The medium was changed by transfer of another bottle containing new HDM after 4 h culture and thereafter at one-day intervals. Cultured conditions were under a humidified atmosphere of 5% CO_2 and 95% air at 37°C .

Figure 1. Detail of drug metabolism simulator (DMS) using PUF/spheroid packed-bed. A: drug injection point; B: sampling point.

Lidocaine metabolic reaction and analytical procedures

Lidocaine metabolic reaction of hepatocytes in monolayer and PUF/spheroid stationary culture were performed in HDM containing 0.25 mM and 1.0 mM lidocaine with a final concentration of 0.5% ethanol (EtOH). The reaction in stationary culture was performed at 2-days intervals of medium exchange on a rotary shaker in 50 rpm under a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. After 24 h, the reacted medium and cells were transferred to a new test tube. The culture dish used for lidocaine metabolism was washed with 200 μ l of EtOH and the washed EtOH was also added to the solution in the new test tube. After addition of 100 μ l 1N NaOH to the solution, 0.25 mM or 1.0 mM *p*-diethoxybenzene dissolved with EtOH as an internal standard for quantitative analysis were also added to the solution. The substrate and metabolic products were extracted with 2 ml of toluene. The organic phase in the test tube was transferred to a vial tube as a sample for gas chromatography/mass spectrometer (GC/MS) analysis.

In the drug metabolic reaction using DMS on 2 days of culture, 0.25 ml of 4 mM-lidocaine dissolved in

EtOH were injected at the drug injection point in DMS containing 50 ml of culture medium (Fig. 1). The initial lidocaine concentration was 20 μ M in DMS. The analysis sample was taken from the sampling point at 10, 20, 30 min, 1, 2 and 3 hr, after lidocaine injection. The samples for GC/MS analysis were extracted according to the procedure described above. In the preliminary experiments, we investigated whether lidocaine itself was not converted to metabolites in the culture medium without hepatocytes for 24 h.

The samples were analyzed by GC/MC (HEWLETT PACKARD 5890, USA). The column of HP-5MS (Crosslinked 5% Ph Me Silicone, ID; 0.25 mm, Length; 30 m, HP, USA) was used for the analysis. Analytical conditions were as follows, initial temp.; 100 °C, rate; 20 °C min⁻¹, final temp.; 270 °C, Inj. temp.; 280 °C. 1 μ l of sample was injected to GC/MS.

Calibration curves were constructed by analyzing only culture medium spiked with the lidocaine and internal standard, according to the procedure described above. Peak area ratios between lidocaine and internal standard were plotted as functions of the concentration in the range from 1 μ M to 1 mM.

Measurement of lidocaine dose-response on cell survival and function in monolayer and PUF/spheroid stationary culture

The cultured hepatocytes in monolayer and PUF/spheroid stationary culture on 5 days of culture were changed into 2 ml fresh medium containing a certain concentration (0 mM ~ 12.5 mM) of lidocaine. The final EtOH concentration of each medium was 0.5%. After the hepatocytes on each culture were exposed to each medium for 24 h, the changes of cell survival and albumin production rate were determined by MTT method (Pearse et al., 1961) and ELISA method, respectively.

The viable cell numbers were by by modified MTT method. MTT was dissolved in PBS buffer at a concentration of 5 mg ml⁻¹. 0.5 ml of MTT solution was added to a cultured dish containing cells and 2 ml of medium in both culture systems. After 12 h, MTT formazan converted from MTT by mitochondria in 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 spectrophotometer (HITACHI U-2000). Calibration curves were constructed by measurement of isolated hepatocytes in a range from 1.0×10^4 to 1.0×10^6 viable cells.

Results

Spheroid formation of hepatocytes and characteristics

When hepatocytes were inoculated into the collagen-coated dish, cells attached and spread within 4 h and then formed a monolayer (Fig. 2A). On the other hand, cells inoculated into the PUF plate attached to the internal surface of PUF pores within 4 h, and then gathered spontaneously and made loose cell aggregates in the PUF pores at 1–2 days of culture (Fig. 2B). Later, rearrangement and compaction of the cells occurred and multicellular spheroids were formed in the PUF pores within a range of 100–150 μ m in diameter after 2–3 days of culture (Fig. 2C). Most of these spheroids adhered to the internal surface of PUF pores and were maintained for more than 2 weeks in stationary cultures.

Lidocaine metabolism of rat hepatocytes in monolayer and spheroid stationary culture

A gas chromatogram of lidocaine metabolites was

Figure 2. Photographs of primary rat hepatocytes in monolayer and PUF/spheroid culture. Hepatocytes plated on collagen-coated culture dish to form a monolayer are shown at day 1 in (A). The development of spheroid form in a pore of PUF is shown at day 1 (B) and day 2 (C). The initial loose aggregates of hepatocytes in the pore of PUF seen at day 1 are gradually increased giving a tightly packed spheroid from around day 2 onward. Bar: 200 μ m.

the same in both cultures (Fig. 3). The metabolites were identified with lidocaine itself and monoethylglycinexylidide (MEGX) by a mass fragment pattern of GC/MS (Fig. 4). The rat hepatocytes in both cultures converted lidocaine to MEGX which was N-

Figure 3. Gas chromatogram of lidocaine metabolism by primary rat hepatocytes. A: PUF/spheroid culture; B: monolayer culture. IS: p-diethoxybenzene. M: MEGX, LC: Lidocaine.

deethylation of lidocaine.

The lidocaine disappearance rates per viable hepatocyte at $250 \mu\text{M}$ lidocaine, which is reported to be apparent K_m of lidocaine de-ethylation reaction in rat liver, were not markedly different until after 2 days in both cultures. In monolayer culture, the lidocaine disappearance rate continuously decreased to one third of the initial level for 10 days, while in PUF/spheroid culture it was maintained at almost the initial level of $30 \mu\text{g}$ 10^{-5} viable cells/day for 10 days (Fig. 5A). The lidocaine metabolizing activity in spheroid culture under 1 mM lidocaine concentration, gradually decreased, but was also higher than in monolayer culture (Fig. 5B).

Toxic effects of lidocaine on cell survival and function of hepatocytes

Lidocaine as a local anesthetic is also known to be a

Figure 4. Mass spectra of lidocaine and MEGX, (A) Lidocaine; (B) MEGX.

toxic substance for hepatocytes. Then, dose response effects of lidocaine on cell survival and albumin secretion activity in monolayer and spheroid culture were investigated.

At the 5th day of culture, the viable cell numbers in lidocaine-free medium were 1.86×10^5 cells in spheroid culture and 1.84×10^5 cells in monolayer culture, which were estimated by MTT method. These cell densities were considered to be 100% cell survival in each culture. The cell survival decreased by lidocaine treatment in a dose dependent manner as shown in Fig. 6A. But the cell survival in PUF/spheroid culture was maintained at a higher level than that in monolayer culture.

The albumin secretion rate in spheroid and monolayer culture on day 5 were $2.95 \mu\text{g}$ 10^{-5} total cells/day and $0.76 \mu\text{g}$ 10^{-5} total cells/day, respectively, which was considered to be 100% albumin secretion activity. These activities also decrease with lidocaine treatment

Figure 5. Difference in lidocaine metabolic activity of hepatocytes in monolayer and PUF/spheroid stationary culture (N = 4). A: difference in lidocaine disappearance rate. B: difference in MEGX appearance rate. Initial concentration of lidocaine: 0.25 mM. ●: PUF/spheroid culture; ○: monolayer culture.

depending on the lidocaine concentration (Fig. 6B). But the activity in PUF/spheroid culture was maintained at a higher level than that in monolayer culture. The ED₅₀ values of albumin secretion were lower than those of cell survival in both cultures.

Lidocaine metabolism by DMS using PUF/spheroid packed-bed

Pharmacokinetic analysis of lidocaine metabolism using PUF/spheroid packed bed was investigated to quantify the activity of drug metabolism simulators

Figure 6. Difference in cell survival and albumin expression changes by lidocaine treatment in monolayer and PUF/spheroid stationary culture. A: changes of cell survival. B: changes of albumin expression. ●: PUF/spheroid culture; ○: monolayer culture.

including hepatocyte/spheroid (N = 4). Administered lidocaine in this device at day 2 of culture was rapidly decreased for 2 h as shown in Fig. 7. After lidocaine metabolism, total cell numbers in this device were $4.00 \pm 0.68 \times 10^7$ cells/module, which corresponded to 0.4 g-liver. The distribution volume of the device was 50 ml. Flow rate of the medium was 4.58 ± 0.05 ml min⁻¹. From this result, the elimination rate constant of lidocaine was $1.51 \pm 0.162 \times 10^{-3}$ min⁻¹. With this device it is possible to evaluate pharmacokinetics in hepatocytes, because this seems to be a small liver model *in vitro*. By using one compartment model, lidocaine clearance and extraction ratio of lidocaine, which was defined as $(C_{in} - C_{out})/C_{in}$, of this drug metabolism simu-

Figure 7. Lidocaine metabolism by DMS device (N = 4). Immobilized cells: $4.00 \pm 0.68 \times 10^7$ cells/module. Elimination rate constant of lidocaine: $1.511 \pm 0.162 \times 10^{-3} \text{ min}^{-1}$. Lidocaine clearance: $0.111 \pm 0.033 \text{ ml/min/module}$. Extraction ratio of lidocaine: $0.0677 \pm 0.0159/\text{g-liver}$.

lator was calculated to be $0.11 \pm 0.033 \text{ ml/min/module}$ and $0.0677 \pm 0.0159/\text{g-liver}$, respectively.

Discussion

It is very important to be able to assess drug metabolism in animals and humans *in vitro*, because, if it becomes possible, a replacement and reduction of animal testing and experimentation will be achieved. These achievements will make it possible to reduce the period and cost for development of new drugs. Several *in vitro* systems are available for assessing the drug metabolism of liver, i.e. cells transfected to express specific human P450, microsomes, liver slices, hepatoma or primary hepatocyte cell culture. When studying the biotransformation of novel compounds *in vitro*, with the aim of predicting the *in vitro* liver-based metabolism of this drug, it would be advantageous to have a model that retains the characteristics of the *in vivo* hepatocyte. Drug metabolism in liver includes several steps, for example, membrane transport of drugs, oxidation/reduction reaction by cytochrome P-450, and conjugation reaction by cytosol enzymes. Therefore, transfected cells and microsomes express only a limited step of the metabolism. Hepatoma cells may vary their drug-metabolizing enzyme activities with time in culture (Doostdar et al., 1990). Liver slices are intend-

ed to maintain the integrity of the three-dimensional microenvironment of primary hepatocytes, but are available for short-term use only and storage of liver slices is even more difficult than cultivation of hepatocytes (Bader et al., 1994).

Conventionally cultured primary hepatocytes in monolayer lose drug metabolizing activities within the first 2–3 days (Bader et al., 1992). This effect was also found in this study (Fig. 5) using lidocaine as a model drug. On the other hand, the activity of hepatocytes/spheroid in 250 µM lidocaine, which is reported to be the apparent K_m of lidocaine de-ethylation reaction in rat liver (Nyberg et al., 1977), was maintained for 10 days at 1.5–2.0 times higher level than that of hepatocytes/monolayer as indicated in Fig. 5A. Under 1 mM of higher lidocaine concentration, the metabolizing activity of spheroid gradually decreased for 10 days, but the level was also higher than monolayer culture. Such a stable maintenance of the activity of primary hepatocytes *in vitro* is very important for assessing drug metabolism in animals and man.

The decrease of lidocaine metabolism at high concentration may be due to a toxic effect of lidocaine. We also investigated the toxic effects of lidocaine on cell survival and function of hepatocytes in spheroid and monolayer culture. Albumin secretion activity was more sensitive to lidocaine than cell survival, and certainly the activity in spheroid was inhibited to about 80% under 1 mM lidocaine for 24 h treatment. But dose responses of cell survival and function in spheroid culture were more tolerant to lidocaine than monolayer culture. These phenomena seem to result from the high metabolizing activity of lidocaine in the spheroid.

Finally, we developed the drug metabolism simulator using a PUF/spheroid packed-bed culture system, which is a device for assessing drug metabolism and pharmacokinetics. By using this simulator, $0.0677 \pm 0.0159/\text{g-liver}$ of extraction ratio of lidocaine was obtained, which corresponded to $1.354 \pm 0.318 \text{ ml/min/g-liver}$ of lidocaine clearance. These values are comparable with *in vivo* values, $0.096/\text{g-liver}$ and $1.930 \text{ ml/min/g-liver}$ which includes an assumption of 20 ml min^{-1} of blood flow rate in the rat liver, reported by Nyberg (1977).

The results described above suggested that hepatocytes/spheroid using PUF as a culture substratum maintains lidocaine metabolism activity as *in vivo* for a comparatively longer time than conventional culture of hepatocytes. Consequently, PUF/spheroid 3-dimensional culture seems to provide a promising cul-

ture system for the development of a drug metabolism simulator.

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