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Behavior of Hepatocytes Inoculated in Gelatin-Immobilized Polyurethane Foam

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Abstract: We have fabricated gelatin-immobilized polyurethane foams (PUFG) by dipping polyurethane foam (PUF) in an aqueous solution containing gelatin and by subsequent reaction with glutaraldehyde after freeze-drying. Gelatin aqueous solutions of different concentrations were used as the dipping solutions to control the amount of immobilized gelatin. The average pore size of PUF decreased with an increase in gelatin concentration. It was found from the hepatocyte adhesion experiment that the amount of hepatocytes seeded on PUFG1, prepared by using a 1% aqueous gelatin solution, was higher than that on other PUFGs. The hepatocytes inoculated in PUFG1 were slightly aggregated as the incubation time increased. The cells inoculated in PUFG1 showed higher ammonia removal ability than those monolayer-cultured on a gelatin-immobilized polystyrene dish (PSG) after 1 and 4 days of incubation time. The inoculated cells exhibited higher albumin secretion relative to monolayer-cultured hepatocytes on PSG. Albumin secretion by hepatocytes seeded on PUFG1 was increased by the presence of serum and was further increased by both the presence of serum and cytokines. The results obtained from a 3-(3,4-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay indicated that PUFG can provide a better microenvironment for hepatocyte culture along with nutrition and metabolite transfer through the high porosity of PUF.

Keywords: gelatin, polyurethane foam, hepatocyte, albumin secretion.

Introduction

Recently, the development of a bioartificial liver (BAL) system has become a real possibility for curing fulminant hepatic failure patients. Immobilizing primary hepatocytes and maintaining their high-level hepatic functions as long as possible are very important for composing a BAL system. Many kinds of polymer substrata have been developed for this purpose. Of the many substrata derived from various materials, the one formed by polyurethane is probably the most common.[1-15]

Funatsu *et al.* have immobilized hepatocytes on polyurethane foams (PUF) with a porosity of 90%. They prepared hepatocyte-inoculated PUFs with different cell densities and studied the morphologies and hepatic functions of the inoculated cells.[4,6,10] They have developed a hybrid artificial liver support system composed of a bioreactor packed with hepatocyte-inoculated PUFs and have evaluated its metabolic performance by applying the system to hepatic failure animals. Their results showed that the level of blood ammonia concentration is more efficiently reduced in the BAL experiment than in the control. Sato *et al.*[16] have immobilized poly-*N*-*p*-vinylbenzyl-lactonamide (PVLA) on PUFs for the long-term maintenance of primary rat hepatocyte culture and have reported that the bioactivity of hepatocytes is improved by supplementing the PVLA as an extracellular matrix. This result suggests that the existence of cell-specific ligands or extracellular signaling molecules can facilitate the interaction of hepatocytes with biomaterials, thus leading to improving the metabolic function of cells in culture.[17-20] Evans *et al.*[21,22] have used gelatin gels for long-term preservation of hepatocytes and have reported that gelatin can provide hepatocytes with a good microenvironment for keeping their hepatic function. The alginate scaffolds have also been used for immobilizing hepatocytes.[23-25] However, their mechanical properties are not enough due to the ionic bond formed during the preparation of the scaffold. Oshima *et al.* have also developed reticulated polyvinyl formal (PVF) resins to immobilize hepatocytes on them.[26-28]

In the present study, gelatin-immobilized polyurethane foams (PUFG) were prepared by dipping the polyurethane

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foams in a gelatin aqueous solution containing glutaraldehyde. Gelatin-immobilized polystyrene dishes (PSG) were also prepared by the treatment of PS dish with oxygen plasma glow discharge, followed by the graft polymerization of acrylic acid and by subsequent coupling reaction with gelatin. The surfaces of PUFG and PSG were characterized using scanning electron microscope (SEM) and electron spectroscopy for chemical analysis (ESCA). The morphologies and metabolic functions of rat hepatocytes adhered to PUFG and PSG were also studied.

Experimental

Fabrication of PUFG and PSG. PUF was kindly donated by Korea Polyol (pore size; 300~500 μm, Ulsan, Korea). The PUF has a three dimensionally reticulated structure with continuous interconnecting pores within its matrix and has porosity higher than 95%. Hybridization of PUF with gelatin was achieved by forming gelatin networks between the interstices of PUF. The circularly sliced PUFs (diameter; 15 mm, thickness; 4 mm) were submerged in and washed with distilled water to remove any chemical contaminants and sterilized with steam autoclave. The PUF was then immersed in a gelatin aqueous solution. To fill the PUF pores with gelatin molecules, we pushed the foam and released it slowly using a pipette. This process was repeated to remove air bubbles from the pores. The PUF filled with gelatins was then taken out, frozen at -72 °C for 24 hrs, and freeze-dried under a vacuum of 0.2 Torr for 24 hrs. The concentrations of gelatin aqueous solution used for the immobilization are in the range of 0.5 to 4%. The family of PUFG is named as PUFG0.5, PUFG1, PUFG2, and PUFG4 depending on the concentration of gelatin aqueous solution. The crosslinking reaction of gelatin molecules in PUF was carried out by the treatment of 25% glutaraldehyde aqueous solution at -4 °C for 6 hrs. After crosslinking, the PUFG was treated with 0.1 M glycine aqueous solution to block unreacted aldehyde groups. Finally, the PUFG was washed with deionized water and freeze-dried.

To prepare PSG, the PS dish with a diameter of 35 mm (Non-treated dish, IWAKI, Japan) was treated by oxygen plasma glow discharge for 30 s with 120 W at 13.56 MHz radio frequency and exposed to air for 10 min to produce peroxides on the surfaces.[29,30] These peroxides were then used as radical initiators for acrylic acid polymerization to prepare carboxylic acid group-introduced PS (PS-C). PSG was prepared by the coupling reaction of PS-C with gelatin in the presence of water soluble carbodiimide.

The surface morphologies of PUF and PUFG were investigated using a scanning electron microscope (SEM, Hitachi S-4200, Japan), and the chemical composition of the surfaces were analyzed for chemical analysis with an electron spectroscope (ESCA, ESCALAB MKII, V. G. Scientific Co., East Grinstead, U.K.) equipped with Mg Kα at 1253.6 eV with 100 W power at the anode. Spectra were taken at an angle of 55°.

Isolation of Hepatocytes. Primary hepatocytes were isolated from female Spraque-Dawley rats (5-7 weeks old, weight 80-200 g, Daehan Biolink Co., Korea) using the modified *in situ* perfusion method.[31] The dead hepatocytes were removed by density gradient centrifugation on Percoll (Amersham Pharmacia Biotech, Sweden). The Percoll solution (1.065 g/mL) used to recover hepatocytes was prepared by diluting Percoll (1.13 g/mL) with Hanks solution. The viable primary hepatocytes were suspended in a Williams E (WE) medium containing penicillin (50 μg/mL) and HEPES (10 mM). Only those isolated hepatocytes with greater than 85% viability by trypan blue dye exclusion were used for the experiments.

Hepatocytes Culture. Freshly isolated hepatocytes suspended in WE medium (5×10^5 cells/mL) were used to inoculate PSG with the cells. The culture medium was replaced daily with a fresh one. The collected medium was centrifuged at 14,000 rpm for 10 min and the supernatant was stored at -72 °C for the subsequent albumin assay. For hepatocyte culture, the PUFGs (diameter; 15 mm, thickness; 4 mm) were placed in a 24-well culture plate and 2 mL of the medium containing 1×10^7 cells was slowly dropped on each PUFG with a pipette. The plate was then incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The residual cell medium was re-dropped on PUFG every hour. After 6 hrs, the medium was replaced with the fresh one to remove the unattached cells. The medium was then replenished daily. The stationary culture was continued for 8 days. By this process, about 7×10^6 cells could be seeded on each PUFG.

Morphological Observation. Hepatocytes inoculated in a PS, PSG, PUF, and PUFG were fixed using a phosphate buffer solution (PBS, pH = 7.4) containing 2.5% glutaraldehyde. The cell-seeded matrix was serially immersed in 25, 75, and 90% ethanol for 15 min, dried in a critical point drier, and finally coated with gold by sputtering. The morphologies of hepatocytes seeded were then examined using a SEM.

MTT Assay. The 3-(3,4-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the reduction of soluble yellow MTT tetrazolium salt to blue MTT formazan product by mitochondrial dehydrogenases, was conducted according to a method previously reported.[32] Briefly, MTT was dissolved in a serum- and antibiotic-free WE medium at a concentration of 0.5 mg/mL and then filtrated to remove any insoluble residue. The PUFG1 inoculated with hepatocytes was placed in a 24-well flat-bottomed plate. The medium containing MTT was then added to a 24-well plate in a volume of 2 mL and incubated for 4 hrs. Thereafter, 2 mL of 0.04 N HCl-isopropanol (1:24, v/v%) was added to the plate after removing the supernatant and kept for 30 min at room temperature in the dark to dissolve the intracellular MTT formazan product. The absorbance

of the formazan solution was read at a wavelength of 570 nm on a kinetic microplate reader, the Multiskan MS (Labsystems, Helsinki, Finland). The calibration curve was constructed by the measuring isolated hepatocytes in a range of 1.0×10^4 to 1.0×10^6 viable cells. In the cell seeding experiment, the number of unattached cells was counted by the MTT method.

Ammonia Removal. To assess the ammonium metabolism of the cultured hepatocytes, a medium loaded with 1 mmol/L ammonium chloride was used at day 1 and days 4. The culture medium was sampled at 0, 2, 4, 6, and 8 hrs after medium exchange. The ammonium concentration in the medium was measured using a commercially available test kit, the Wako Ammonia Kit (Indophenol method; Wako Pure Chemical Industry, Osaka, Japan).

Albumin Synthesis. The amount of albumin in the culture medium collected at various time intervals was measured by means of enzyme-linked immunosorbent assay (ELISA)[32] using rabbit anti-mouse albumin serum (Inter-Cell Technologies, Inc., Hopewell, NJ, USA) and sheep anti-mouse albumin polyclonal antibody (The Binding Site, Birmingham, UK). Peroxidase-conjugated anti-rabbit IgG (Dako A/S, Denmark) and orthophenylenediamine (OPD, Sigma) solution (3 mg/mL OPD and 0.02% H_2O_2 in citrate-phosphate buffer, pH 4.8) were used as the secondary antibody and peroxidase substrate, respectively. Absorbance of the solution at 492 nm was measured using a kinetic microplate reader. The albumin concentration of the sample was calculated from the calibration curve constructed with rat albumin of known concentrations as standards.

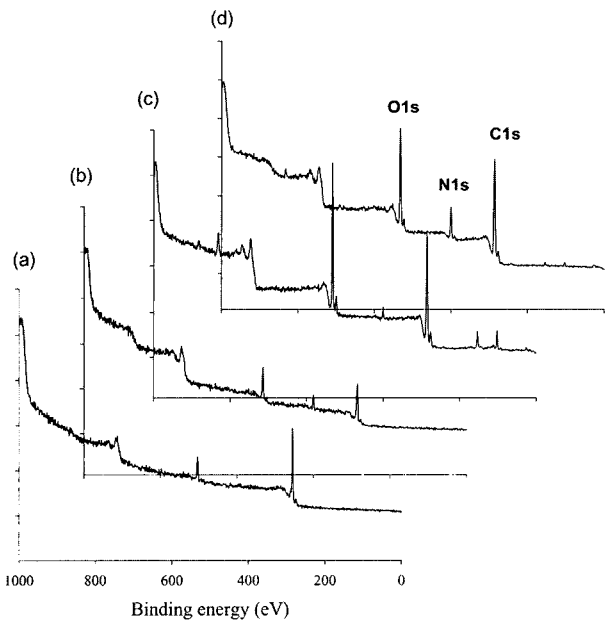

Figure 1. ESCA survey scan spectra of (a) PS, (b) PSG, (c) PUF, and (d) PUFG1.

Results and Discussion

Surface Characterization. The changes in chemical composition of the gelatin immobilized PS dish and PUF surfaces were investigated using ESCA. Figure 1 shows ESCA survey scans of (a) PS, (b) PSG, (c) PUF and (d) PUFG surfaces. PS surface showed two peaks corresponding to C1s (285 eV) and O1s (532 eV) while PUF showed an additional peak at 400 eV ascribed to N1s. In the case of PUF, however, two peaks appeared at 107 eV (Si_{2p}) and 159 eV (Si_{2s}). This is due to the contamination with silicone compounds. Chemical compositions of the surface-modified PSs and PUFs, calculated from the ESCA survey scan spectra, are shown in Table I. The oxygen content (11.9%) of the PS surface was increased by gelatin immobilization (19.4%). On the other hand, the oxygen content (27.9%) of PUF was decreased by gelatin immobilization (22.4%). An increase in the percentage of nitrogen on the PSG (11.4%) and PUFG (11.0%) surface is attributed to the high nitrogen content of the gelatin itself. The SEMs representing the morphology of gelatin-immobilized PUFs are shown in Figure 2. The porosity of PUF gradually decreased with an increase in the gelatin concentration employed for immobilization reaction. This result indicates that the pores of PUF were gradually blocked by the crosslinked gelatin network.

Adhesion of Cells to PSG. To demonstrate the effect of gelatin on cell adhesion, hepatocytes were cultured in PS and PSG for 6 hrs. As shown in Figure 3, cells were poorly adhered to the PS control while largely adhered to the PSG. This result indicates that gelatin immobilization can facilitate hepatocytes adhesion onto the PSG surface due to the introduction of arginine-glycine-aspartic acid (RGD) residues of gelatin.

Morphology of Hepatocytes Inoculated in PUFG. To determine the optimum condition of gelatin immobilization, hepatocytes were seeded on PUFGs with different amounts of gelatin and their results are shown in Figure 4. Cells were almost not adhered to the PUF control. This is because PUF has lower wettability and a low affinity for hepatocytes adhesion. The highest packing ratio of cells (ca. 70%) was found on PUFG1, which was prepared by using 1% gelatin solution. Cell adhesion was largely suppressed on PUFG4. In PUFG4, cells cannot be penetrated into the pores, thus

Table I. Elemental Composition of Surface-Modified PSs and PUFs Calculated from ESCA Survey Scan Spectra

Substrates	Atomic Percent (%)		
	C	O	N
PS	88.1	11.9	-
PSG	69.2	19.4	11.4
PUF	69.7	27.9	2.4
PUFG	66.6	22.4	11.0

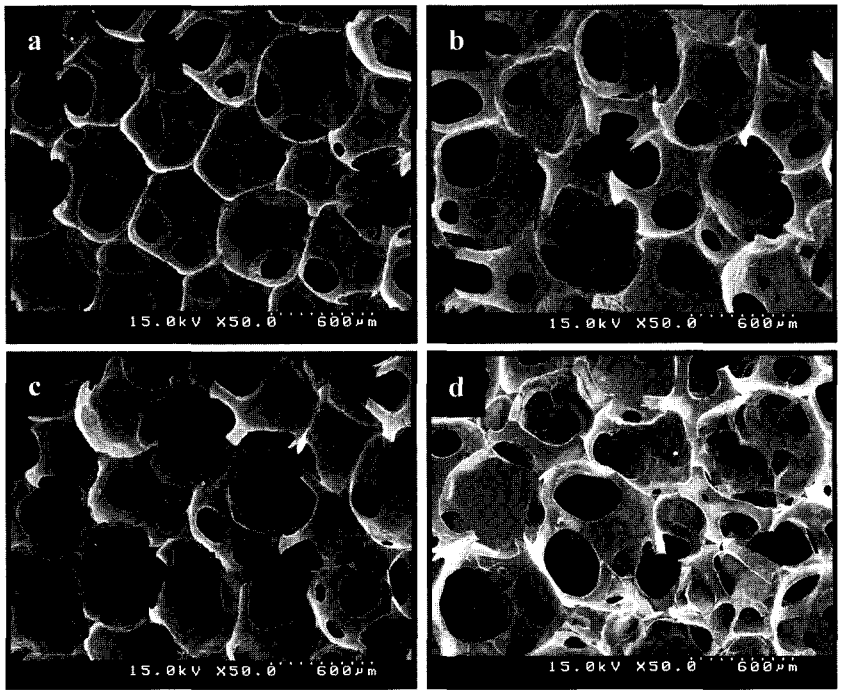

Figure 2. SEM photographs of the surface of hybridized PU foams; (a) PUF, (b) PUFG0.5, (c) PUFG1, and (d) PUFG2.

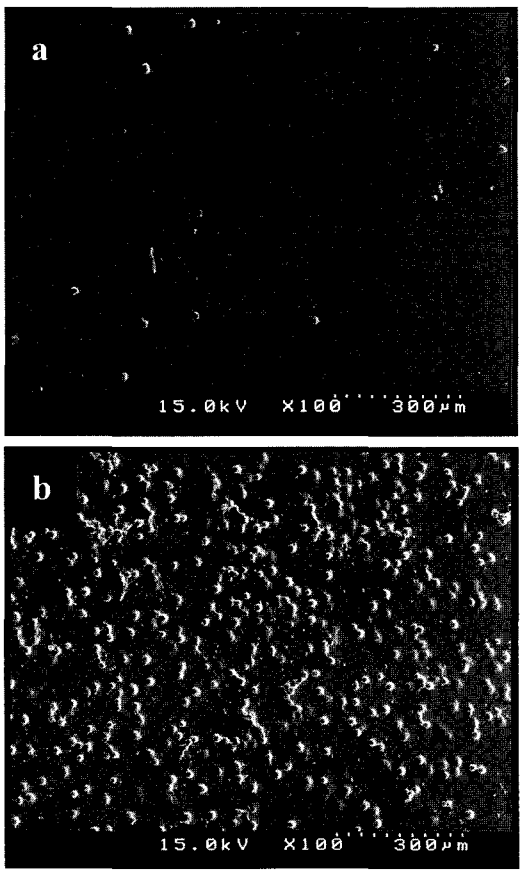

Figure 3. *SEM photographs of the hepatocytes adhered to the surface-modified PS dishes after 6 hrs of incubation; (a) PS and (b) PSG.*

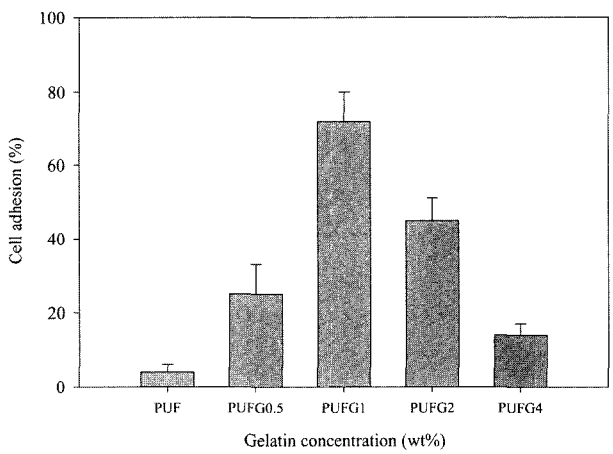

Figure 4. Adhesion of hepatocytes to gelatin-hybridized PUFs after 6 hrs of incubation.

leading to the decrease of cell packing ratio. It is considered that the cell packing ratio is influenced by both the porosity and pore shape in a three-dimensional polymer matrix. Kurosawa *et al.*[6] have used a polyurethane membrane with a porosity of 90% and finger-like structure macropores for an efficient immobilization carrier for high-density culture of rat hepatocytes. From this result, PUFG1 was used for further experiments. In the following hepatic function tests, all the biochemical assays were performed upon the hepatocytes inoculated in PUFG1.

Figure 5 shows SEM microphotographs of hepatocytes adhered to gelatin hybridized PUF after 1 day of incubation.

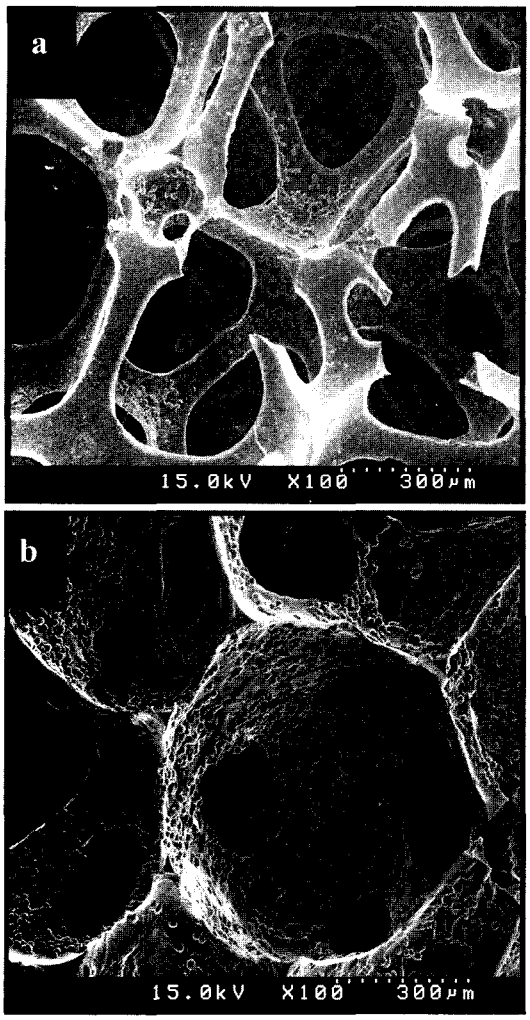

Figure 5. SEM photographs of the hepatocytes adhered to gelatin-hybridized PUF surface after 1 day of incubation; (a) PUF and (b) PUFG1 (original magnification; 100×).

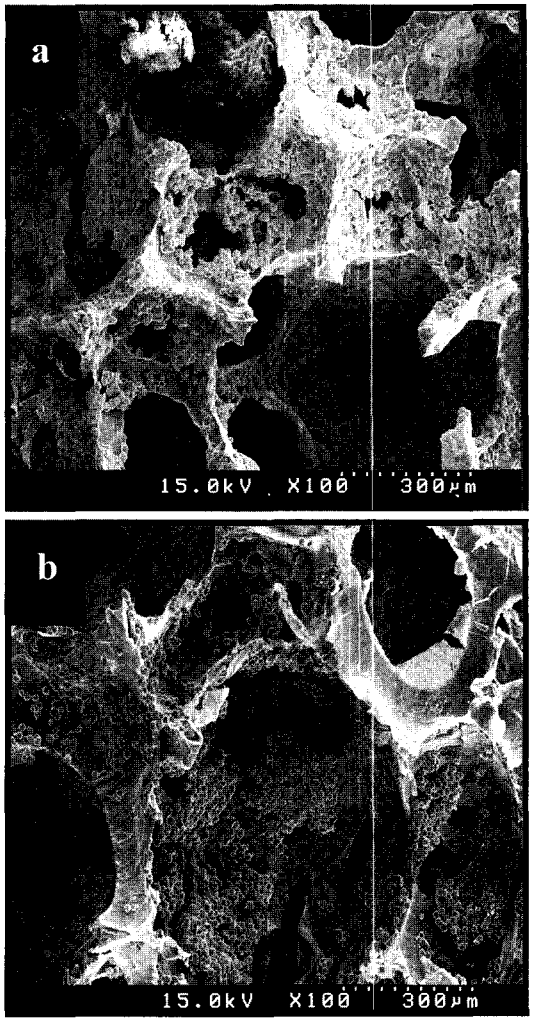

Figure 6. SEM micrographs of the hepatocytes adhered to PUFG1 after 8 days of incubation; (a) surface and (b) cross-section (original magnification; 100×).

Hepatocytes were largely adhered to PUFG1 while less adhered to PUF control. After 8 days incubation, the hepatocytes aggregated on the both surface and cross section of PUFG1 as shown in Figure 6. There was no obvious difference in cell density on the surface and cross-section of PUFG1. Funatsu *et al.*[14] have observed the spherical muticellular aggregates (spheroids) of adult rat hepatocyte which spontaneously formed in the pores of PUF. However, in the current study, only slight aggregation was formed on PUFG1.

Longevity of Hepatocytes Inoculated in PUFG. During 8 days culture of the hepatocytes inoculated in PUFG1, cell viability was estimated using the MTT method every second day and their results are shown in Figure 7. The initial MTT activity of hepatocytes inoculated in PUFG1 (0 day) was fixed at 100 and used as the control. It can be seen, in the first 4 days, that the mitochondrial activity of hepatocytes

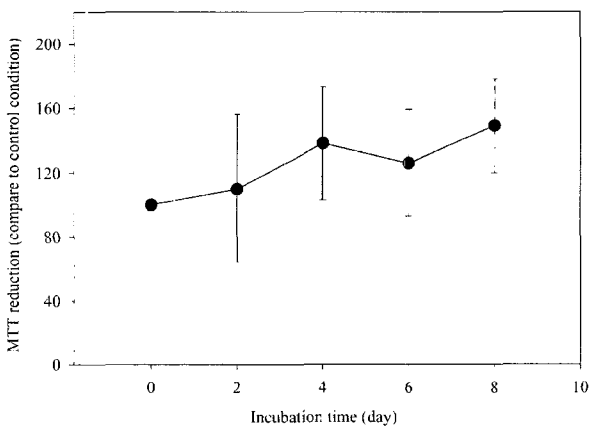

Figure 7. MTT reduction obtained from the hepatocytes adhered to PUFG1 as a function of incubation time. The absorbance of the formazan solution obtained from the hepatocytes precultured for 6 hrs was taken as 100.

inoculated in PUFG1 slightly increased with culture time compared to the initial state (100%), and then kept this level to days 8 (149%). This result indicates that the hepatocytes almost recovered their mitochondrial activity in 4 days. The MTT results obtained in this study suggest that PUFG can provide a microenvironment, resulting in the recovery of mitochondrial activity of inoculated hepatocytes.

Metabolism of Hepatocytes Inoculated in PUFG. In order to evaluate the detoxic function of hepatocytes, ammonia removal by hepatocytes cultured in PSG and PUFG for 1 day was carried out and the results are shown in Figure 8. It can be seen that ammonia removal by the hepatocytes adhered to PUFG1 (●) was higher than for those adhered to PSG (○). The ammonia removal pattern of hepatocytes cultured in PUFG1 for 4 days was almost the same as that for 1 day (data were not shown). On the other hand, after 4 days, ammonia removal ability was not detected upon monolayer hepatocytes due to the cell detachment. This indicates that PUFG1 provides a better condition for hepatocyte culture than PSG. The ammonia removal ability of hepatocytes cultured on PUF1 for 4 days was about 0.09 μmol/h/10^{6}cell. Ohshima *et al.*[26] immobilized hepatocytes on reticulated PVF resin, and reported that the immobilized hepatocytes showed a 0.3 μmol/h/10^{6}cell of ammonia removal ability.

The albumin secretion by hepatocytes inoculated in PUFG1 was measured in different culture media and their results are shown in Figure 9. It can be seen that the hepatocytes inoculated in PUFG1 showed higher albumin secretion ability than those cultured on PSG under the same medium (WE medium + fetal calf serum (FCS)). This result indicates that PUFG1 provided a suitable condition for albumin secretion compared to PSG. The albumin secretion ability of inoculated hepatocytes might be improved by the supplement of cytokines.[33] The hepatocytes cultured in the medium supplemented with FCS and cytokines (■, epidermal growth factor (EGF) + insulin) showed the highest albumin secretion ability. The hepatocytes cultured in a medium containing EGF and insulin showed intermediate albumin secretion ability. Similar results have been reported elsewhere.[6] In the presence of cytokines, albumin secretion by the cells increased up to 6 days and thereafter slightly decreased.

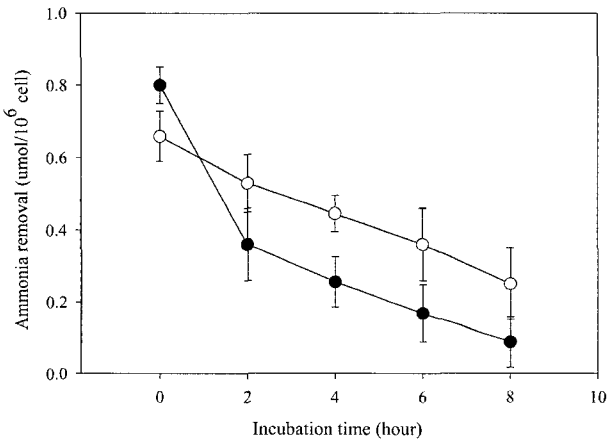

Figure 8. The change of ammonia concentration in the medium which was caused by rat hepatocytes in PUFG1 after 1 day of culture; (●) PUFG1 and (○) PSG. The samples were collected each 2 hrs (0.2, 4, 6 and 8) and the medium was changed with fresh W.E. medium after the assay. Data expressed as mean ± S.D. (n=3).

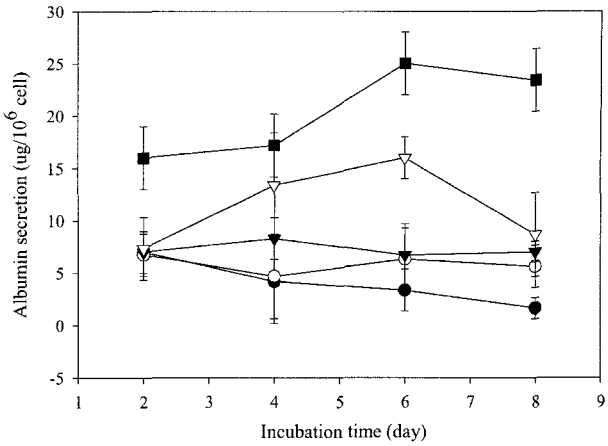

Figure 9. Albumin secretion by rat hepatocytes cultured on PSG and PUFG1. The culture medium was replaced with the fresh one every day and the amount of albumin secreted into the medium by heaptocytes for 24 hrs was assayed by means of ELISA. Data expressed as mean ± S.D. (n=3); (●) PSG (+FCS), (○) PUFG1 (medium), (▼) PUFG1 (+FCS), (▽) PUFG1 (+EGF, insulin and dexamethasone), and (■) PUFG1 ((+EGF, insulin, dexamethasone and FCS).

Conclusions

Gelatin immobilized PUFs were prepared and used as substrates for the inoculation of rat hepatocytes. Immobilization of gelatin was confirmed using ESCA and SEM. PUFG1, which was prepared by the reaction of PUF and 1% gelatin solution, was best for the achievement of hepatocytes immobilization. Hepatocytes aggregation was observed after 8 days of culture. It was found from the MTT results that hepatocytes inoculated in PUFG1 kept a high mitochondrial activity for 8 days of culture. It was also revealed from metabolic evaluation that hepatocytes inoculated in PUFG1 showed not only relatively good ammonia removal ability, but also albumin secretion. The albumine secretion by hepatocytes further increased with the addition of cytokines.

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