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Effects of Degree of Cell-Cell Contact on Liver Specific Functions of Rat Primary Hepatocytes

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Abstract Cell-cell interaction and the extracellular matrix (ECM) are believed to play essential roles during *in vitro* culturing of primary hepatocytes in the control of differentiation and in the maintenance of tissue specific functions. The objective of this study was to examine the effects of degree of cell-cell contact (DCC) on liver specific function of rat primary hepatocytes Hepatocyte aggregates with various degrees of cell-cell contact, *i.e.,* dispersed cells, longish aggregate, rugged aggregate, and smooth spheroid were obtained at 1, 5-6, 15-20, and 36-48 hrs, respectively in suspension cultures grown in spinner flasks embedded in Ca-alginate bead and collagen gel in order. The smooth spheroids displayed a decrease in viability and functional activities. This may result from mass transfer limitation and shear damage caused by agitation during aggregation. The rugged aggregate showed a higher viability and albumin secretion rate than the dispersed cells or the other aggregates. This result indicates the possible enhancement of a bioartificial liver's (BAL) performance using primary hepatocytes and the reduction in time to prepare a BAL through optimization of the immobilization time.

Keywords: collagen, alginate, cell-cell interaction, hepatocyte aggregate, hepatocyte spheroid

INTRODUCTION

Since hepatocytes are responsible for the maintenance of the body's metabolic homeostasis, including catabolism, storage, protein synthesis, biotransformation, detoxification, and excretion of bile [1], there is much interest in their use with in the studies of drug metabolism, toxicology, gene expression and the bioartificial liver support system. In particular, application of BAL has developed by the recent tissue engineering technology [2] It is an inherently interdisciplinary field that applies the principles of life sciences, biomaterials, bioengineering, and clinical sciences towards the development of a biological substitute to restore, maintain, or improve tissue function [3] The BAL will act as a bridge to provide patients with more time until a donor organ becomes available for transplantation or until their own livers can be regenerated [4].

Hepatocytes have a distinctive epithelial polarity and cell-cell communication structures, including bile canaliculi, a tight junction, and a gap junction, and also show high regeneration and differential functions [5-7]. However, they lose these characteristics when cultured *in vitro*. Therefore there have been many difficulties in the *in vitro* culturing of hepatocytes. The research done to date on the long-term culture of hepatocytes can be classified as follows. Firstly, the optimization of culture media components, such as growth factors, *i.e* epidermal growth factor (EGF) and hepatocyte growth factor (HGF), various hormones secreted by the pancreas, such as insulin and glucagon, as well as a variety of nutrients and minerals, have been carried out [8-10]. Secondly, the optimization of the components in the extracellular matrix (ECM) including types I, III and IV collagens, fibronectin, laminin, *etc.*, and the culture systems, such as the collagen coated dish, membrane supported sandwich and gel embedding systems, have also been carried out [8,11-14]. Due to their anchorage dependency, cell-matrix interactions have also been intensively investigated in hepatocyte cultures. In several experimental systems, it has been well documented that the ECM controls cellular adhesion, migration, proliferation, differentiation, apoptosis, as well as cytokine and growth factor production [8,15-16]. Thirdly, the optimization of cell-cell interactions has also been showed Spheroid cultures using a homogeneous cell-cell interaction and co-cultures utilizing heterogeneous cell-cell interactions have been studied along these lines [17-20]

When hepatocytes are cultured as spheroids, cell-cell contacts occur inside the aggregate and the hepatocytes show a greater differentiation and have more functions than monolayer cultures [17,18,21,22]. However, little research has been done on the effect of degree of cell-cell contact (DCC) on maintaining the differentiated functions of the hepatocytes. Patricia and Saltzman (1993) used such small aggregates, having only 4-6 hepatocytes each, that they could not investigate the effect entirely [23]. Surapaneni *et al* (1997) examined a broad range of aggregate size, however, their aggregation method using a tissue culture flask generally required a large surface area to form large quantities of aggregates for BAL construction [24]

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In this study we were able to take easily large quantity of hepatocyte aggregates with various DCC by controlling the time that suspension cultures of the hepatocytes were grown. Our intention was to investigate the effects of the DCC on hepatocyte viability and liver specific functions by immobilizing the aggregates in either Ca-alginate or a collagen gel

MATERIALS AND METHODS

Hepatocytes Isolation

Hepatocytes were isolated from adult Sprague-Dawley rats weighing 180 to 200 g by a two-step *in situ* collagenase perfusion technique modified from the method described by Seglen [25,26]. The rat was first anesthetized with xylazine (1 mg/100 g body weight, Bayer Korea Ltd., Seoul) and ketamine hydrochloride (5 8 mg/100 g body weight, Yuhan Corp., Seoul) Following midline incision and cannulation of the portal vein, the liver was perfused *in situ* with 250 mL of perfusion buffer I for 10 min The perfusate was oxygenated by first passing it through the inside of an oxygen-permeable silicone tubing, which was placed in an appropriate chamber containing 90% oxygen and 10% CO_2, at a flow rate of 25 mL/min Air bubbles in the buffer solution were removed by a bubble trapper. After the initial flush out, perfusion buffer II supplemented with collagenase (hepatocyte-qualified, Gibco BRL, Grand Islands, NY, USA) and trypsin inhibitor (Gibco BRL) was perfused at a flow rate of 20 mL/min for 10 min. The constitutions of perfusion buffer I and perfusion buffer II are given in Table 1. After 10-20 min, upon visual and palpable evidence of the liver dissolving, the capsule was broken and the liver substance was raked and irrigated with cold Williams' medium E (Gibco BRL). The released cells were filtered through a nylon mesh, with 100 μm openings, and resuspended in fresh Williams' medium E. The cell suspension was centrifuged (500 rpm, 50 g, 2 min) and the pellet was resuspended in Williams' medium E. This centrifugation and resuspension step was repeated. Finally, the cells were counted in a hemacytometer and viability was assessed by the trypan blue exclusion method The average yield was from 2 to 3 × 10^8 cells with a viability in excess of 85%

Culture of Hepatocytes as Spheroid

The culture medium employed for all cultures was Williams' medium E (Gibco BRL) supplemented with the following additives: epidermal growth factor (20 μg/L, Gibco BRL), insulin (10 mg/L, Gibco BRL), $CuSO_4$ $5H_2O$ (0.1 μM, Sigma Chem. Co., St. Louis, MO, USA), $ZnSO_4$ $7H_2O$ (50 pM, Sigma Chem. Co.), H_2SeO_3 (3 μg/L, Sigma Chem Co), linoleic acid (50 mg/L, Sigma Chem. Co.), $NaHCO_3$ (1.05 g/L, Gibco BRL), HEPES (1 19 g/L, Sigma Chem. Co), penicillin (100 Unit/mL, Gibco BRL), streptomycin (10 mg/mL, Gibco BRL), and amphotericin B (0.25 μg/mL, Gibco BRL). In the rest of the text this medium will be referred to as hormonally defined medium (HDM).

The freshly isolated hepatocytes were then resuspended in HDM at a concentration of 5 × 10^5 cells/mL. The cell suspension was placed in siliconized spinner flasks (250 mL, vertical paddle, Bellco Glass Inc., Vineland, NJ, USA) and stirred by a magnetic stirrer (Bellco Co.) at 60 rpm in a humidified 95% air/5% CO_2 incubator at 37°C. After 8 and 24 h of culturing, the medium was replaced with fresh HDM Hepatocytes growing in the spinner flask gradually aggregated and finally formed spherical aggregates within 48 h.

Table 1. Compositions of perfusion buffers used for rat hepatocyte isolation

Compositions	Buffer I (g/L)	Buffer II (g/L)
NaCl	8	8
KCl	0.4	0.4
$CaCl_2$	-	0.56
$NaH_2PO_4 \cdot 2H_2O$	0.078	0.078
$Na_2HPO_4 \cdot 12H_2O$	0.151	0.151
HEPES	2 381	2.381
Collagenase	-	0 5
Trypsin inhibitor	-	0.05
EDTA	0 19	-
$NaHCO_3$	0 35	0.35
Glucose	0.9	-
Penicillin	100 (unit/mL)	100 (unit/mL)
Streptomycin	0.1	0 1

Immobilization of Hepatocytes

Immobilization of the hepatocytes were performed four times, at 1, 5-6, 15-20 and 36-48 h, with the suspension cultures using the Ca-alginate bead and collagen flat gel methods. For the former method, a 2% (w/v) sodium alginate solution was prepared with ultra pure water and sterilized by autoclaving. This was then mixed with 2 × medium (1:1, v/v) to make a 1% (w/v) solution. Hepatocytes from the spinner flask were mixed with the alginate solution at a density of 1 × 10^6 cells/mL and were dropped into a vigorously stirred $CaCl_2$ solution, consisting of 100 mM $CaCl_2$ (Sigma Chem. Co), 10 mM HEPES (Sigma Chem Co.), and 20 mM D-fructose (Sigma Chem Co.), through a 21G blunt end needle with an air flow rate of 16 L/min. Under these conditions, the size of the Ca-alginate beads ranged from 1,000 to 1,500 μm After stirring for 30 sec, the beads were meshed and washed with PBS (Phosphate buffered saline) and placed into a 6-well plate (Deep-well, 35 mm, Nunc, Denmark). The plates were then shaken on an orbital shaker (Barnstead/Thermolyne, Dubuque, IA, USA) that was placed in a CO_2 incubator. One million hepatocytes were seeded in each well with 2 mL of HDM. The medium was changed daily.

Collagen flat gel type was employed using Tissue Culture Inserts (Nunc, diameter 25 mm, 3.0 μm pore polycarbonate membrane), which provide the gel and hepatocyte aggregates with media and oxygen both from the top and bottom. Type I collagen (Sigma Co.) was sterilized by UV illumination for 20 min and then prepared as a 0.3% (w/v) solution in 0.05% acetic acid. Hepatocytes with different DCC were suspended in a

collagen gel solution (8 parts collagen solution: 1 part 10 × medium: 1 part buffer containing 50 mM NaOH, 20 mM HEPES, and 25 mM $NaHCO_3$) at 1.5×10^6 cells/mL of cell density. This solution was then pipetted into the insert (1 mL/insert), placed in a 6-well plate and then incubated for 30 min to accelerate gelation. Subsequently, 3 mL of HDM was added and the medium was changed daily

After 2 days, HDM containing 1 mM ammonium chloride was used to assess the ammonia removal activity of the hepatocytes. Spent medium was stored at −20°C prior to analysis of the liver specific functions

The Measurement of Hepatocytes Viability

MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide, Sigma Co.) analysis method [27] was used to check the viability of the hepatocytes at the end of each culture period The spent medium was removed and the cells were suspended at a concentration of 5×10^5 cells/mL in 0.03% (w/v) MTT solution in PBS followed by incubation for 3-4 h The MTT analyzing medium was removed and isopropyl alcohol (Oriental Chemical Co., Seoul, Korea) acidified with 0 04 N HCl (Junsei Chemical Co., Tokyo, Japan) was added to give 5×10^5 cells/mL. This extraction was done at the room temperature for 4 h. Absorbance of the extracted solution was read at 570 nm in an UV-VIS spectrophotometer (Smart plus 2605, Young Hwa Co., Seoul, Korea).

Measurement of Ammonia Removal and Urea Secretion Activity

To assess the ammonium metabolism and urea synthesis activity of the cultured hepatocytes, medium loaded with 1 mM ammonium chloride was used after 2 days of culturing. Ammonia and urea concentrations in the media were measured colorimetrically using commercially available test kits, *i.e.*, the Asan Blood Ammonia Assay Kit (Asan Pharma., Korea) and Blood Urea Nitrogen Assay Kit (Sigma Co. No636) [19].

Measurement of Albumin Concentration

Collected media samples were analyzed for rat albumin content by the sandwich enzyme-linked immunosorbent assay (ELISA) method as previously described [14]. Antibodies to the albumin and pure rat albumin were purchased from Cappel (#55727, 55952, 55776, Durham, NC, USA). Results were quantitated at 490 nm wavelength with an ELISA reader (Ceres UV900-HDi, Bio-Tek Instrument). The concentrations of samples were determined from a standard curve generated for each ELISA plate Generally, two duplicate wells were averaged for each sample.

RESULTS

The Morphology of Hepatocytes Cultured as a Suspension, Immobilized in a Ca-alginate Gel Bead and in a Collagen Flat Gel

Hepatocytes cultured as a suspension in the spinner flask formed multicellular spherical aggregates within 48 h These spheroids exhibited a tightly packed morphology and smooth surfaces The aggregation process is shown in Fig. 1. The initial cell suspension contained a considerable amount of hepatocyte doublets and triplets (Fig. 1(a)) and longish aggregates of twenty to thirty cells per aggregate appeared at 5-6 h culture (Fig. 1(b)). Irregular rugged aggregates and smooth surface spheroids formed within 15-20 (Fig 1(c)) and 36-48 (Fig. 1(d)) h in suspension culture, respectively. The size of the final aggregates was about 170-240 μm [19]. As shown in Fig. 1, the DCC increases rapidly as the initial cells aggregate to final spheroids The names given to the aggregates, in order, are dispersed cell (DC), longish aggregate (LA), rugged aggregate (RA), and smooth spheroid (SS).

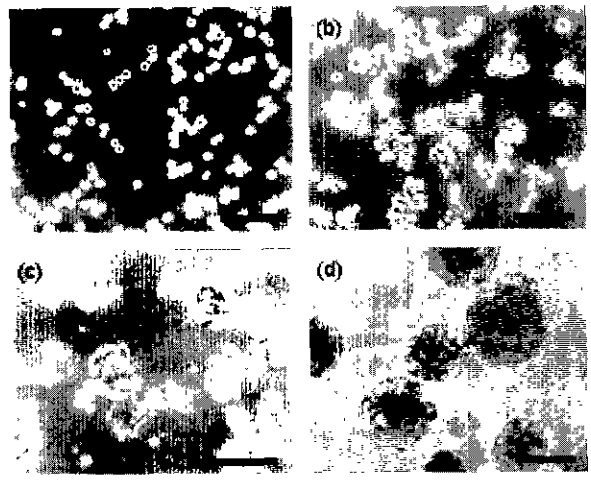

Fig. 1. Light microscopic pictures of hepatocytes cultured in a suspension in a spinner flask, (a) dispersed cells (DC, 1 h), (b) longish aggregates (LA, 5-6 h), (c) rugged aggregates (RA, 15-20 h), (d) smooth spheroids (SS, 36-48 h) Scale bar indicates 200 μm.

To investigate the effects of DCC, hepatocyte aggregates were taken four times from the spinner culture at different culture times, *i.e.*, 1 (DC), 5-6 (LA), 15-20 (RA) and 36-48 (SS) h, and the aggregates were immobilized in either a Ca-alginate bead or a collagen flat gel as previously described.

When DC and SS were embedded in the Ca-alginate bead, both the aggregates maintained their original shapes during the culture periods (Fig. 2(a) and 2(c)). On the other hand, the bumpy surfaces of LA and RA gradually became round in shape. In any case, integration between neighboring aggregates did not occur.

In contrast, DC and the other aggregates immobilized in the collagen flat gel showed quite different morphogenetic responses. All had flat and extended shapes, which were presumed to be the result of cell-matrix interactions. Unintentional cell-cell contact occurred considerably in case of DC, which was caused by a disproportionate dispersion of the cell population due to cell settlement during gelation of the collagen (Fig. 2(b)). After immobilization within collagen gel, large aggregates, such as RA and SS, began to extend dendritic processes from the aggregates into the surrounding matrix within a few days (Fig. 2(d1)-(d4)). This

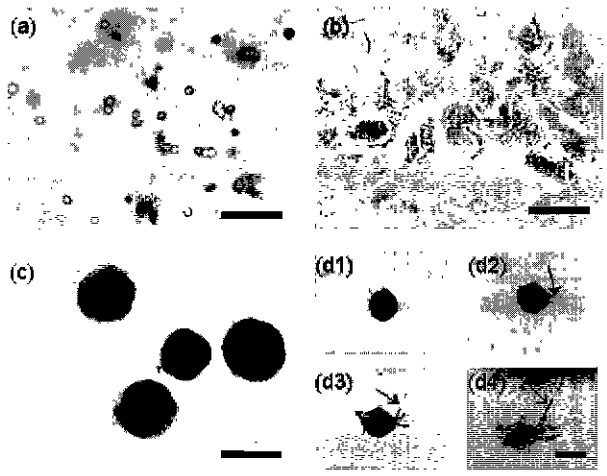

Fig 2. Light microscopic pictures of hepatocytes immobilized in a Ca-alginate gel bead (a and c) and a collagen flat gel (b and d) While DC and SS immobilized in the collagen gel underwent considerable changes in morphology (b and d), those in the Ca-alginate gel kept their initial shapes (a and c). Photographs (d1) through (d4) are of the same spheroid. Arrows indicate the dendritic processes presumed to be a bile duct-like structure (a) DC, 5 days, Ca-alginate, (b) DC, 5 days, collagen, (c) SS, 5 days, Ca-alginate, and (d1-4) SS, 2, 5, 6, and 10 days, collagen Bars indicate 200 μm.

morphogenesis was observed in most of the SS and in some of the RA and continued throughout the culture period.

Liver Specific Functions of Immobilized Hepatocytes

To investigate the influence of the DCC on liver specific functions of hepatocytes immobilized in the Ca-alginate bead, ammonia removal and urea and albumin secretion rates were determined Fig 3 shows the functions of hepatocytes in the Ca-alginate beads DC, LA and RA efficiently removed the ammonium ion from the medium for up to a week while the ammonia removal activity for SS was about a half of DC's. RA showed stability for the first six days but showed a relatively lower removal rate than DC and LA on the 7th day Similar trends were also shown in the urea secretion rates (Fig. 3(a) and 3(b)). In the urea secretion rate, however, the difference between RA and SS narrowed gradually due to an increase in SS's rate at the fifth day. Significant differences in the functional activity between the various hepatocyte aggregates were seen with albumin secretion (Fig. 3(c)). For the DC, albumin secretion rate decreased gradually and dropped to nearly zero by day 7 In contrast, RA showed higher secretion rate than that of any other aggregates during the week LA also showed a relatively higher albumin secretion during the first three days, but the activity declined rapidly from the fourth day. The initial secretion rate of SS was consistently lower than LA and RA, but remained more stable during the latter part of the experiment.

Hepatocyte aggregates embedded in collagen gels

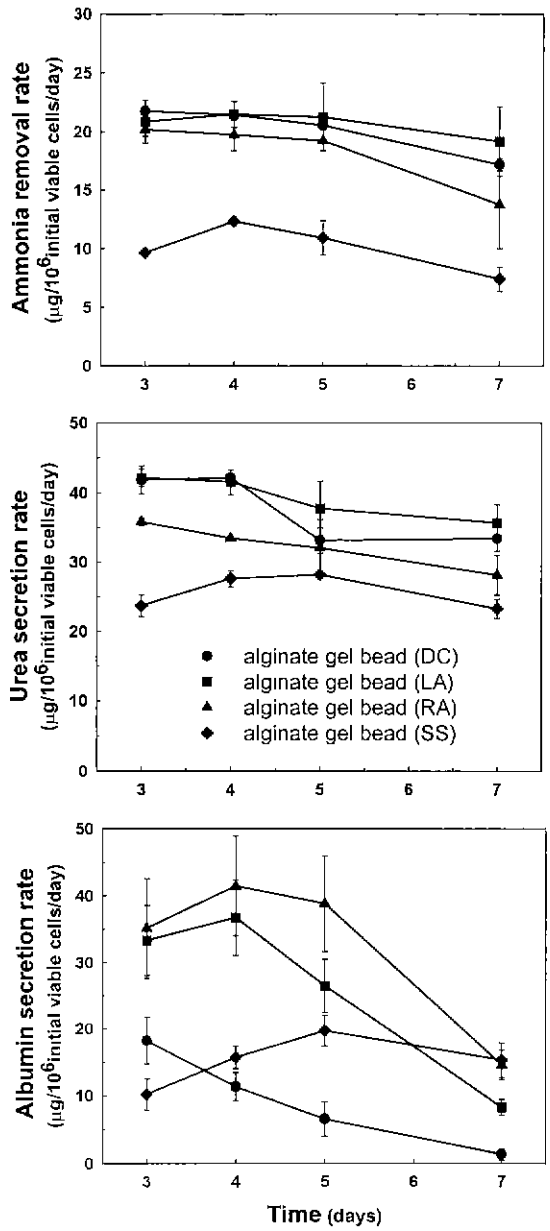

Fig. 3. Effect of the DCC on liver specific functions, such as ammonia removal (a) and, urea (b) and albumin (c) secretion rates, for the hepatocytes immobilized in the Ca-alginate bead Data are shown as the mean ± the standard deviation for four cultures from two independent isolations.

showed similar trends for urea secretion and ammonia removal rates with those in the Ca-alginate beads (data not shown). On the other hand, albumin production of the hepatocytes in the collagen flat gel was found to be quite different. Unlike the striking influence of the DCC in the Ca-alginate system, all the hepatocyte aggregates (DC, LA, RA and SS) in the collagen gel secreted a similar amount of albumin into the media at an unstable level of about 20 μg/10^6 cells/day throughout the 7-day culture period (data not shown)

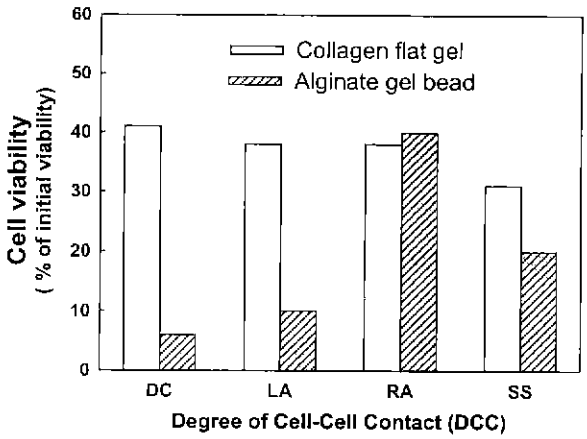

Fig 4. Effect of the DCC on the viability of the hepatocytes immobilized in a Ca-alginate bead and a collagen flat gel. MTT viability assays was performed at 17 days of culture. Data shown is the average of four cultures from two independent isolations

Hepatocytes Viability

To assess the influence of cell-cell contact on the viability of the cultured hepatocytes, an MTT assay was performed after 17 days of culturing for both immobilization systems. The viability was expressed as a percentage of the initial activity Each data point represents the average of four samples from two independent isolations. When hepatocyte aggregates were cultured in Ca-alginate beads, they showed significant differences in viability. As shown in Fig 4, the viability of RA was the greatest with it being 2, 4 and 6 times higher than SS, LA and DC, respectively

For the collagen flat gel system, viabilities of DC, LA and RA were similar to each other, while SS showed a somewhat lower viability.

DISCUSSION

Hepatocytes, major constituents of the liver lobules, are large polygonal cells forming one-cell-thick plates along sinusoids without definite support of a collagenous matrix. By sandwiching hepatocytes between two layers of a type-I collagen gel, an *in vivo* microenvironment was closely mimicked *in vitro*. The culture configuration was simply constructed by overlaying the hepatocyte monolayer on the collagen gel with another collagen gel Within this configuration, the hepatocytes maintained liver specific activities, such as the secretion of albumin and urea, for much longer periods than those cultured on a single layer of collagen gel [8,11]. Hepatocyte spheroids, tightly packed multicellular aggregates, have been observed to exhibit enhanced liver specific activities and a prolonged differentiated state compared to cells that were maintained as a monolayer [28], however, they have an excess cell-cell contact in comparison with *in vivo* structure or the sandwich system. Due to its dense organization, a mass transfer limitation may be occuring at the center of the spheroids [29], especially when an immobilization method is used for the construction of a BAL. In spite of this disadvantage, very few works have been done on the optimization of DCC and the development of a scale-up method of the sandwich system.

In this study we used a spinner flask for efficient formation of a large quantity of hepatocyte spheroids. Hepatocyte aggregates with various DCC, labeled DC, LA, RA, and SS, were obtained during this process and these were immobilized using conventional methods, namely Ca-alginate beads and collagen gels. By measuring the liver specific activities of these aggregates we investigated the effects of DCC and found the appropriate DCC for BAL system.

When hepatocytes and their aggregates were embedded within the collagen gel matrix, they exhibited a flat and extended morphology. This phenomenon was also observed when the cells were cultured on the top of collagen gel layer and indicates an active expression of cytoskeletal proteins, such as actin, tubulin and cytokeratins [30]. These kind of morphogenetic responses are undesirable in the aspect of maintenance of liver specific functions, because it has been revealed that an inverse relationship between cytoskeletal and liver-specific protein expression exists [30]. The correlation was well-grounded by the fact that hepatocytes cultured on Matrigel, which was found to be the most appropriate extracellular matrix (ECM) extracted from the Engel-breth-Holm-Swarm (EHS) mouse tumor, attached rapidly but exhibited minimal spreading and an elevated stable liver specific protein production [8,31].

It was reported that hepatocyte spheroids embedded within a collagen gel and then cultured with a medium containing both epidermal growth factor (EGF) and insulin extended many dendritic processes, which were positive for cytokeratin 19, a marker for bile duct cells [15]. Identical behavior was observed in our study (Fig. 2(d1)-(d4)) and the phenomenon will provide a useful tool for investigation of morphogenesis *in vitro*. As mentioned above, however, the response was undesirable for the expression of the liver specific functions. Therefore, additional studies on the effect of matrix and media components will be needed to control the gene expression of the hepatocytes

Hepatocytes and their aggregates settled down to the bottom of the insert which supported the collagen flat gel with porous membrane during about 20 min of a gelation period. As a result, the cells were all on one side of the porous membrane and, therefore, a much higher cell-cell contact resulted than intended, especially in the cases of DC and LA (Fig. 2(b)). As a consequence, the diversity in the DCC between DC, LA, RA and SS were severely reduced, so that it was very difficult to evaluate the influence of the DCC on the liver specific activities of the hepatocytes cultured within the collagen flat gel. Indeed it was found that there was little difference in the albumin secretion rates between the four different kinds of aggregates (data not shown). If we could disperse the aggregates evenly inside the gel by eliminating the unintentional increase in cell-cell contact after embedding in collagen gel, the problem mentioned above would be removed.

SS showed lower ammonia removal and urea secretion rates as well as viability, as measured by MTT conversion, than those of RA in the Ca-alginate bead system (Fig 3(a), 3(b)). It was speculated that the Ca-alginate shell, and also the spheroid itself, act as a barrier against mass transfer or that the spheroids were already damaged by the shear stress caused by initial agitation for 48 h in the spinner flask In general, urea secretion and ammonia removal activities of hepatocytes are affected little by the DCC while the albumin secretion activity is strongly affected by DCC Since BAL treatment of patients with fulminant hepatic failure lasts less than 8 h [32,33], initial functional activities of the hepatocytes are the core element for development of the liver support device. Judging from the above criteria, LA and RA have more proper DCC for manufacturing a BAL system than what DC and SS have. This would mean that it would not be necessary to wait until complete spherical aggregates are formed, which takes about 2 days Therefore, the preparation time for a BAL system can be reduced considerably, that is, from two days to less than 15 h.

The viabilities of hepatocytes cultured in Ca-alginate beads and collagen flat gels for 17 days clearly indicate that the cells are strongly anchorage-dependent *i e.* require attachment to a solid substrate or other cell membrane for survival and function DC in the Ca-alginate beads lost most of its albumin secretion activity and viability by 7 and 17 days respectively (Fig. 3(c) and Fig. 4), which was due to the inadequacy of Ca-alginate gel to promote cellular attachment. Interestingly, all the aggregates embedded within collagen gel except SS exhibited a viability as high as RA in the Ca-alginate beads. Therefore, it was proven once again that hepatocytes need a good matrix surface for their attachment. The relatively low viability of SS in both systems can be explained again by what was previously mentioned *i e.*, the mass transfer resistance and the shear damage.

The results presented in this paper indicate that the use of longish and rugged aggregates of hepatocytes formed in a spinner vessel will enhance the performance and reduce the construction time of a BAL system.

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