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Phenotype of Hepatocyte Spheroids in Synthetic Thermo-reversible Extracellular Matrix

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Abstract Aggregates of specific cells are often regarded as a better form in artificial organs and mammalian cell bioreactors in terms of cell-specific functionality. In this study, the morphology and liver-specific functions of freshly harvested primary rat hepatocytes, which were cultivated as spheroids and entrapped in a synthetic thermo-reversible extracellular matrix, were examined and compared to a control (hepatocytes in single cell form). A copolymer of N-isopropylacrylamide (98 mole % in feed) and acrylic acid (poly(NiPAAm-co-AAc)), a thermo-reversible copolymer gel matrix, was used to entrap hepatocytes either in spheroids or single cells. During a 7-day culture period, the spheroids maintained higher viability and produced albumin and urea at a relatively constant rate, while the single cell culture showed a slight increase in cell numbers and a reduction in albumin secretion. Hepatocytes cultured as spheroids present a potentially useful three-dimensional cell culture system for application in a bioartificial liver device.

Keywords: artificial liver, thermo-reversible extracellular matrix, hepatocytes, spheroids

INTRODUCTION

Currently, whole liver transplantation is the only widely practiced clinical treatment for liver failure and inborn genetic defects. The liver is one of the most important organs, playing essential roles not only in chemical detoxification but also in the metabolisms of sugars, amino acids and lipids. However, the profound shortage of donor organs, the risk and expense involved in surgery, and post-surgical morbidity have recently motivated several research groups to develop hepatic tissue engineering to improve clinical outcome [1-3].

Biohybrid artificial organs represent an important investigational mode in medicine and tissue engineering, as a means for replacing the many metabolic functions of a failed organ. The technique involves culturing cells in purified natural extracellular matrices (ECMs), such as collagen, fibronectin, and laminin *in vitro* and, if necessary, physically immunoisolating them using an artificial membrane. The biohybrid artificial organ can be used as a device *ex vivo* or implanted into patients to replace various functions of a damaged organ/tissue. Optimal cell delivery represents a major challenge requiring the provision of an optimum environment that mimics natural conditions in order that proper cellular functions are adequately maintained. Of the various proposed approaches, cell entrapment in a matrix is probably the most promising approach. The matrix stabilizes cultures by holding the cells in a 3-dimension. A hydrogel, which absorbs considerable amounts of water within a polymeric network, but without dissolving in the water, and which maintains its three-dimensional stability, is considered as an ideal matrix. Both artificially and naturally derived hydrogels provide tissue-like 3-dimensional mesh or porous spaces that support the cells [4], while allowing the transport of wastes, nutrients, other essential molecules, and secreted cell products via the bulk fluid phase [5]. Cells entrapped in such hydrogels can be utilized in *in vitro* applications, such as bioreactor, and can be used in *in vivo* studies, where the hydrogel matrix protects transplanted cells from mechanical and immunological damage.

Several investigators have found that freshly isolated primary hepatocytes can be cultured to form 3-dimensional, tightly packed, freely suspended, multicellular aggregates or spheroids [6,7]. This specialized cell structure has been demonstrated to have enhanced liver specific activities and a prolonged differentiated state compared to cells that were maintained as a monolayer [8,9], and cells in spheroids appear to have similar morphologies and ultrastructure to those found in native liver lobules [10,11]. The ability of hepatocytes to self-organize into a three-dimensional structure is believed to contribute to their enhanced liver-specific activities [8].

A biohybrid artificial liver for chronic liver failure, or temporal liver support for acute liver failure, often requires an extracelluar matrix that can physically prevent further aggregation of hepatocyte spheroids and provide anchorage sites for the cells. Because the hepatocyte functionality in an artificial liver sharply declines with time, the hepatocyte replacement in the artificial

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liver could be an option for extending the lifetime of a device.

In this study, we investigated the use of thermo-reversible poly(NiPAAm-co-AAc) gel as an matrix for hepatocyte spheroids. This particular matrix has been developed as an effective extracellular matrix for the design of a rechargeable cell delivery device, and makes it possible to replenish malfunctioning cells with freshly isolated or cryopreserved cells [12,13] by manipulation temperature around 37°C. The behavior and function of the hepatocyte spheroids entrapped on the poly(NiPAAm-coAAc) matrix were investigated.

MATERIALS AND METHODS

Isolation of Hepatocytes

Hepatocytes were isolated from Sprague-Dawley rats, weighing 200-250 g, by a liver perfusion method using 0.05% collagenase (type IV) [14,15]. More than 99% of the isolated cells were parenchymal as determined by a phase-contrast microscopy and more than 90% were viable as determined by trypan blue dye exclusion method [16]. The cells entirely stained with trypan blue were defined as dead cells.

Thermo-reversible Copolymer Synthesis

A high molecular weight copolymer of NIPAAm and AAc (2 mole %) was synthesized in benzene (10 wt% monomer in benzene) with AIBN as the initiator (7×10^{-3} mol AIBN/mol monomer) and purified as reported in our previous publications [12,13]. In brief, the monomer solution was first purged at room temperature for 15 min using dry nitrogen prior to the addition of the initiator. Polymerization was accomplished at 60°C for 16 h under a nitrogen atmosphere. During polymerization, the copolymer precipitated. After the polymerization had been complete, the benzene was decanted and the copolymer dried and then dissolved in an acetone/methanol mixture (90:10). The copolymer so prepared was precipitated from the acetone/methanol solution into a ten-folds excess of diethyl ether. The poly(NiPAAm-co-AAc) was finally dried and purified by dialysis with a 50,000 molecular cutoff (MWCO, Spectra Por) membrane against distilled water for 96 h, changing the water every 24 h. The chemical structures of the poly(NiPAAm-co-AAc) copolymer gel is shown in Fig. 1.

Cell Culture

Isolated hepatocytes were dispersed in hormonally defined medium (HDM), containing William's E medium supplemented with 10mg/mL insulin (Sigma, St. Louis, MO), 0.1 mM copper ($CuSO_4 \cdot 5H_2O$), 3 nM selenium (H_2SeO_3), 50 pM Zinc ($ZnSO_4 \cdot 7H_2O$), 50 ng/mL linoleic acid (Sigma), 100 U/mL penicillin, 50 mg/L streptomycin, and 1 mg/mL fungizone at a density of 3

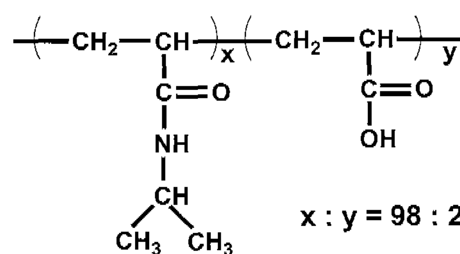

Fig. 1. Chemical structure of poly(NiPAAm-co-AAc).

$\times 10^5$ cells/mL and plated in positively charged plastic dishes (Falcon Primaria 3803, Beckon Dickson, Oxnard, CA). Within 3 days of stationary culture at 37°C under 5% CO_2 and 95% air, all the inoculated cells aggregated to form floating multicellular spheroids of 100-200 cells.

Hepatocyte Spheroids in the Thermo-reversible Hydrogel

Spheroids from the 4th day cultures were entrapped in the gel as follows: Spent medium, 1.5 mL, containing ca. 100-200 floating spheroids (approximately 100,000 cells), was aspirated by a pipette and centrifuged at 500 rpm for one min. Spheroids collected were resuspended in 1.5 mL of medium and mixed with an autoclave sterilized aqueous polymer solution (typically 7 wt%, 300 μL), at room temperature. The cell suspension in the polymer solution was placed in a 12 mm diameter Millicell, with a pore size of 12 μm, and then cultured in RPMI 1640 in 5% CO_2 and 95% air at 37°C. At this temperature the polymer solution became a gel.

Nonspheroidal hepatocytes (single cells) were also entrapped in the gel at the same cell density as a control.

Viability of Hepatocytes in the Gel

After liquefying the gel by lowering the temperature to ambient temperature the entrapped cells were collected before and after incubation of cells by centrifugation and observed by phase-contrast microscopy. Before calculating the cell viability of spheroids, the spheroids were reseeded onto collagen-coated styrene dishes to make the culture of the spheroids as a monolayer state. After monolayer state of cells, the cells were detached and the viability of cells was calculated by trypan blue method. The viability of the aggregates and single cells were assessed by trypan blue dye exclusion under a phase-contrast microscope. Aggregates or single cells entirely stained with trypan blue were defined as dead.

Measurement of Liver Specific Functions

To evaluate the albumin productivity of the cells, the cell suspensions were collected daily and centrifuged (34 g, 2 min) to separate the supernatant from the cell pellet. The supernatants were stored at -20°C until albumin assay, and the spent media were replaced com-

pletely every 2-3 days with fresh culture media. Albumin concentration was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) as follows: Samples were serially diluted, and peroxidase conjugated rabbit antibody against rat albumin was added to a final concentration of 400 ng/mL. After incubation at 37°C for two hours, 100 μL aliquots of each sample were transferred to an albumin-precoated 96-well plate. The precoated plates were prepared adding 100 mL of rat albumin (100 ng/mL in phosphate buffered saline (PBS)) to each well. Overnight incubation at 4°C was followed by washing three times with 0.05% Tween 20 in PBS. After being transferred to the precoated plates, the samples were incubated at room temperature for 2 h in a humidified chamber, the plates again washed three times with 0.05% Tween-20 in PBS and then filled with 100 μL per well of a solution containing 55 mg/mL of 2.2'-azino-di-[3-ethylbenthiazoline-6-sulfonate] in citrate buffer (0.1 M sodium citrate, pH 4.2, and 0.1 mL of 30% hydrogen peroxide). The plates were further incubated at room temperature in a humidified chamber for 30-45 min and read on an automated fluorescence microplate reader. The difference in absorbance at 405 and 492 nm was correlated to the concentration of rat albumin in the sample. Each sample was assayed in duplicate.

Urea nitrogen levels were measured using a diagnostic kit using an urease/Berthelot, quantitative, colorimetric method [11]. 100 mL of sample and 0.3 mL urease solution were added to test tubes. After gentle mixing, the tubes were allowed to stand at room temperature for 15-20 min while the urea was hydrolyzed to ammonia. 0.6 mL of phenol nitroprusside solution, 0.6 mL of alkaline hypochlorite solution, and 1.5 mL of deionized water were then added with gentle vortexing after each addition. After color development, approximately 30 min at room temperature, 200 μL from each test tube was pipetted into a 96-well Falcon microtiter plate. Triplicate samples were analyzed for absorbance at 540 nm on an automated microplate reader. The ammonia concentration was obtained using an urea nitrogen standard curve.

RESULTS AND DISCUSSION

Cell Entrapment

The molecular weights and compositions of the polymers in this study, as determined by conventional characterization techniques, have been previously described [13]. Fig. 2 illustrates each characteristic phase of the copolymer gelation process in an aqueous polymer solution (5 wt%) in phosphate buffered saline (PBS; pH 7.4, ionic strength (I)=0.2 M) with increasing temperature. The clear solution clouds at 29°C (cloud temperature) and subsequently becomes immobile, due to gel formation, and without a significant gel induction time at 35°C (gelation temperature). Initially, the gel formed is translucent but becomes more opaque as

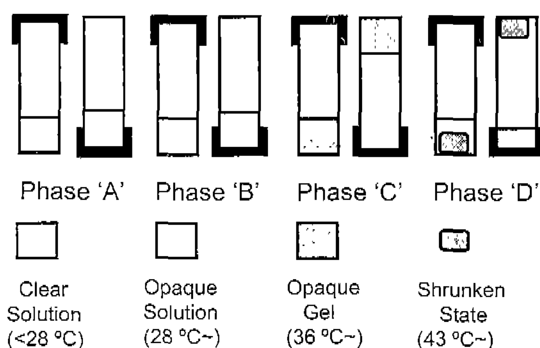

Fig. 2. Temperature-sensitive phase transitions of aqueous poly(NiPAAm-co-AAc) solution.

the temperature is increased. Above 43°C (gel shrinking temperature), the gel starts to shrink. The gelation process is completely reversible with respect to temperature, and hysteresis free. Freshly isolated primary rat hepatocytes were preincubated at 3×10^5 cells/mL in the culture media. During following 24 h period, the cells started to aggregate into increasingly larger cell clumps that eventually formed into tightly packed spherical cell structures. The aggregation process began with the initial aggregation of dispersed cells into doublets and triplets within 2-3 h of inoculation. Larger aggregates (> five cells per aggregate) appeared in the culture at 6 h. Within 24 h, the formation of spheroids, defined as freely suspended cell aggregates that assembled into tightly packed spherical structures with smooth boundaries, was observed. This formation rate was significantly more rapid than that of spheroids formed at least 3 days. The spheroids formed using method exhibited morphologies, which were quite different from cell clumps, in which cells were observed to be loosely connected and irregularly shaped. This observation suggests that the spheroids grew in size, presumably by the coalescence of smaller spheroids over a period of 3 days (data not shown). No remarkable morphological changes were observed in the spheroids during 72 h culture, as determined by phase-contrast microscopy.

In order to entrap the cells in the gel, an aqueous polymer solution in culture medium (pH 7.4, ionic strength; 0.15 M) was mixed with hepatocyte spheroids or single cells and poured into Millicells maintained at 36°C; this temperature caused the solution to form a gel.

We investigated the behavior of hepatocytes in copolymer gels of various concentrations, in the presence of 10 ng EGF/mL, 100 nM insulin and 10% FBS. The 7% solution was found to be optimum in terms of gelation and cell viability. The viability of hepatocyte spheroids cultured this copolymer gel for 7 days showed considerable viability (almost 90%) (Fig. 3), whereas single cell hepatocytes under the same conditions exhibited lower viability (*ca.* 65%). It has been reported that the hepatocytes cultured in an anchorage-independent condition undergo apoptosis [17]. However, these results show

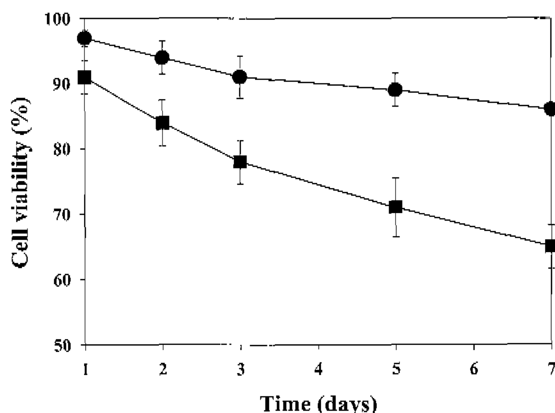

Fig. 3. Viability of spheroids and single cells in the copolymer gel. ●: spheroids, ■: single cells. The data represent a mean ± SD (n=5).

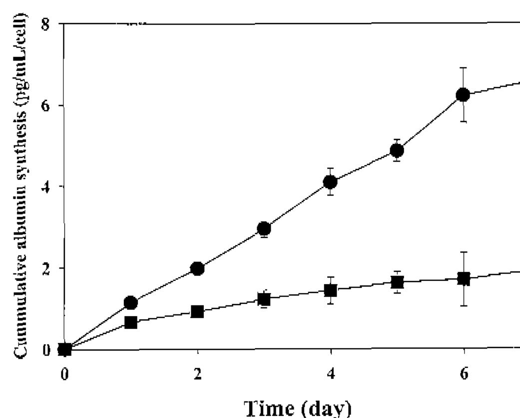

Fig. 4. Albumin synthesis of hepatocyte spheroids and single cells during three-dimensional culture in copolymer gel. ●: spheroids, ■: single cells. The data represent a mean ± SD (n=5).

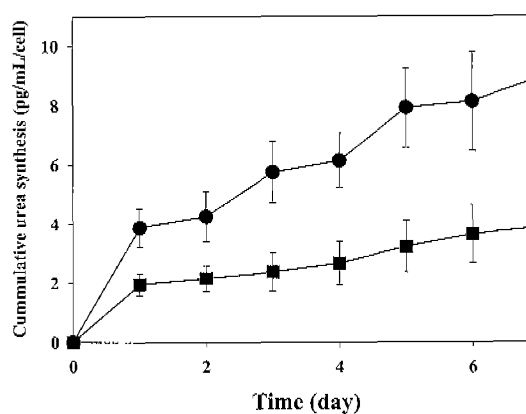

Fig. 5. Urea synthesis of hepatocyte spheroids and single cells during 3-dimensional culture in the copolymer gel. ●: spheroids, ■: single cells. The data represent a mean ± SD (n=5).

that under appropriate culture conditions, hepatocytes remain viable in anchorage-independent culture conditions.

Liver-specific Functions

One of the primary goals of cell entrapment in the gel is to sustain the cell-specific functions of cultured cells. Thus, we chose albumin productivity as an indicator. In order to evaluate albumin release, the solutions of poly(NiPAAm-co-AAc) in medium (7 wt%) were adjusted to pH 7.4 using 1 N NaOH and mixed with the spheroids or single cells. Throughout the 7-day culture period, high albumin biosynthesis was detected at relatively constant rates. An analysis of albumin levels demonstrated that the spheroids were significantly more active in this respect than single cell culture even after a period of one week (Fig. 4), which showed that the spheroids had enhanced liver-specific activity compared to the single cell cultures in the copolymer gel. This feature might reflect upon the potential of hepatocyte spheroids for numerous cell immobilization applications. This result re-confirmed that the spheroidal form of hepatocytes are a better form than single cells for high performance bioreactors or as artificial liver, although the preparation of the spheroids is rather complicated.

Urea secretion from the hepatocytes in multicellular spheroids was also compared with that of cells in single cell gel culture. The cells in multicellular spheroids exhibited both a higher level of urea secretion and longer-term maintenance of function than those in single cell culture. Since the expression of differentiated functions and the proliferative ability are known to be a related reciprocally [18], it is believed more likely that cells in multicellular spheroids experienced stable differentiated states, similar to those in the liver by forming spheroids. The spheroid forms of hepatocytes suggest that these structures may mimic the cellular organization of the liver lobule. Entrapment may provide sufficient exchange of nutrients and other materials between spheroids. Moreover, cell morphology dependent upon cell-cell and cell matrix interactions, and these interactions influence the expression of differentiated functions and proliferative ability.

CONCLUSION

Hepatocytes cultured as spheroids in a thermo-reversible extracellular matrix exhibited higher viability and enhanced liver-specific functions and seemed to better maintain their differentiated state than single cells state under the same conditions. Primary hepatocytes cultured as spheroids are more appropriate for various types of artificial livers. In addition, the use of a thermo-reversible matrix may offer us a cell-rechargeable system and provide us with a useful tool for the study of liver regeneration process and for the development of hepatic cell based bioreactor processes.

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