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Analysis of cell viability and differential activity of mouse hepatocytes under 3D and 2D culture in agarose gel

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Abstract

Three-dimensional (3D) and two-dimensional (2D) cultures of hepatocytes in various concentrations (0.3–0.7%) of agarose gel revealed that the hepatocytes under 3D cultures in 0.3% agarose gel possess long-term (>3 weeks) viability, significant self-assembly to form tissue like aggregates, low lactate dehydrogenase release and high albumin synthesis. These were in contrast to 2D culture of hepatocytes. Our results suggest that the 3D culture of hepatocytes in agarose gel favors aggregate formation of functionally active cells and would be useful for liver transplantation as well as to analyze hepatocytes biology.

Introduction

In tissue engineering, cells are cultured normally on synthetic or purified extracellular matrixes (ECMs), such as collagen, fibronectin, and laminine, etc., *in vitro* and subsequently implanted *in vivo* to guide new tissue formation and to replace all functions of the damaged organ/tissue (Mooney *et al.* 1992, 1994). A major challenge of tissue engineering is to provide an optimum environment that mimics natural conditions in order to maintain proper cellular functions. For example, to obtain a highly efficient bio-hybrid artificial liver, it is important to culture the hepatocytes, the major populations of liver cells, under culture conditions that confers long-term viability of cells and intercellular communications to construct a biomimic tissue with normal cells functions (Hansen *et al.* 1998).

Although both 2D and 3D cultures of hepatocytes on different ECMs are possible, it is reported that the hepatocytes in 3D culture of collagen and Matrigel maintain high differential state (Tong *et al.* 1990, Berthiaume *et al.* 1996). One possible interpretation could be that multicellular aggregates or spheroids formation *in vitro* are important for long-term viability and proper cell–cell communications to maintain high differential state. Since strong cell–ECM interaction partly inhibits spheroid formation *in vitro* under 2D culture, in 3D culture, due to low concentration of ECM, cells can move more easily to form aggregates. For example, hepatocytes aggregates are formed on low affinity substratum, e.g., PVLA-coated surfaces (an artificial asialoglycoprotein model polymer-lactose-carrying polystyrene as a culture substratum) by self-assembly under certain conditions (Kobayashi *et al.* 1994). Furthermore, these aggregates are reminiscent of tissue structure including the formation of junctional complexes between cells and microvillar structures similar to bile canilicular channels (Wu *et al.* 1996). Therefore, it is important to explore the influences of 2D and 3D culture of hepatocytes in an inert surface.

In this study, we demonstrate the effects of 2D and 3D culture of mouse primary hepatocytes in different concentrations of agarose gel on cell viability and functions. Agarose gel is bio-compatible and shows good permeation to form a three dimensional scaffolding of cells. Like other ECMs, it does not have strong interaction (except physical interaction) to cells. Our results reveal that the 3D culture of hepatocytes in agarose gel is more bio-competiable to maintain long-

term viability and self-assembly than they do on 2D culture.

Materials and methods

Cell preparation

Mouse primary hepatocytes were isolated from livers of male ICR mice (7–9 weeks old) (SLC, Shizuoka, Japan) by the modified *in situ* perfusion method as described previously (Morita *et al.* 1995) in detail with slight modifications. Briefly, the dead parenchymal hepatocytes were removed by density gradient centrifugation on Percoll (Pharmacia, Piscataway, NJ, USA). The viable parenchymal hepatocytes were suspended either in William's E medium (Gibco BRL, New York, USA) containing antibiotics (50 μg penicillin/ml, 50 μg streptomycin /ml, and 100 μg neomycin/ml). Isolated hepatocytes, with purity more than 98%, were used in this study.

Cell culture

Agarose-LGT (Nacalai tesque Inc. Kyoto, Japan) was dissolved in water to prepare the gels. An equal volume of 2× William's E medium was added when the gel temperature reached around 37 °C for 2D and 3D culture of hepatocytes in the presence of 10% (v/v) fetal bovine serum (FBS) (Gibco BRL, New York, USA), 100 nM insulin (Sigma, MO, USA), and 10 ng epidermal growth factor (EGF)/ml (Hitachi Chemical Co., Tokyo, Japan). First, agarose gel (0.3–0.5%) with serum and growth factors was pored on the polystyrene culture dishes and allowed to gel formation. This lower gel will prevent the direct contact of hepatocytes with the culture plates. For cell culture, hepatocytes either in William's E medium or in 0.3–0.7% agarose gel along with FBS, EGF, and insulin were seeded on the first layer of agarose gel to perform 2D and 3D culture, respectively. For 3D culture the first layer gel was always 0.5%. A monolayer culture of hepatocytes 2.5×10^4 cells/cm^2 was also performed on polystyrene dishes.

Lactate dehydrogenase (LDH) release assay

LDH released in the culture medium was measured after 72 h using CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, USA). 50 μl culture medium of hepatocytes was mixed with 50 μl substrate solution, incubated for 10 min at 25 °C and the reaction was stopped by adding 50 μl 1.0 M acetic acid. The absorbancy at 492 nm was measured by using a micro-plate reader (Corona electric Co. Ltd., Ibaraki, Japan). [LDH release (%) = 100 × Experimental LDH release from damaged cells/Maximum LDH release from total cells after sonication.] Maximum LDH release was obtained by sonication of the same number of cells used in all experiments.

Viability test by Alamer Blue staining of hepatocytes

Hepatocytes and aggregates in agarose gel were stained with 10% (v/v) Alamer Blue (Iwaki Glass Co., Tokyo, Japan). Alamer Blue stains only the viable cells red by its reducing reaction on mitochondria. Alamer Blue staining was done for 4 h at 37 °C. The stained hepatocytes and aggregates in agarose gel were observed by a confocal laser scanning microscope (Leica, Germany).

Measurement of albumin synthesis

The level of albumin in the medium was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) using rabbit anti-mouse albumin serum (Inter-cell Technologies Inc., Hopewell, NJ, USA) and sheep anti-mouse albumin polyclonal antibody (The Binding Site Ltd., Birmingham, England). The secondary antibody was peroxidase conjugated antirabbit IgG (Seikagaku Co., Tokyo, Japan). Orthophenylenediamine (Wako Pure Chemical Industries Ltd., Osaka, Japan), that absorbs light at 492 nm, was used as the substrate of peroxidase. Absorbancy at 492 nm was measured by using a micro-plate reader.

Results and discussion

Formation of self-assembly aggregates in or on agarose gel

We investigated the behavior of hepatocytes in the various concentrations of agarose gels, all in the presence of 10 ng EGF /ml, 100 nM insulin and 10% FBS (Figure 1). For 3D culture, the concentration of agarose gel was changed between 0.3 to 0.7%. In 0.3% agarose gel, hepatocyte aggregates began to form after 24 h. After 72 h, hepatocyte aggregates increase in size with the increased number of viable hepatocytes (Figure 1). On the other hand, in 0.5 and 0.7% gels, hepatocyte aggregates are hardly formed after 72 h. Hepatocyte aggregation is promoted by EGF (Nishikawa *et al.*

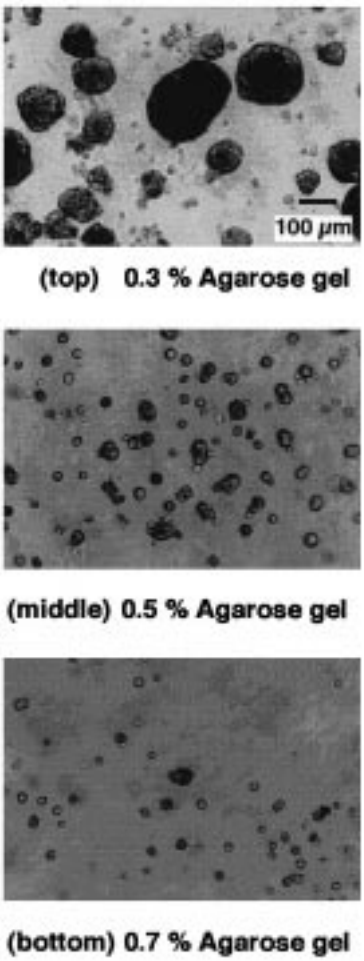

Fig. 1. Behavior of hepatocytes cultured in the various concentrations of agarose gel after 48 h in the presence of 10 ng epidermal growth factor/ml, 100 nM insulin, and 10% fetal bovine serum; (top) 0.3% agarose gel, (middle) 0.5% agarose gel, and (bottom) 0.7% agarose gel. Bar = 100 μm

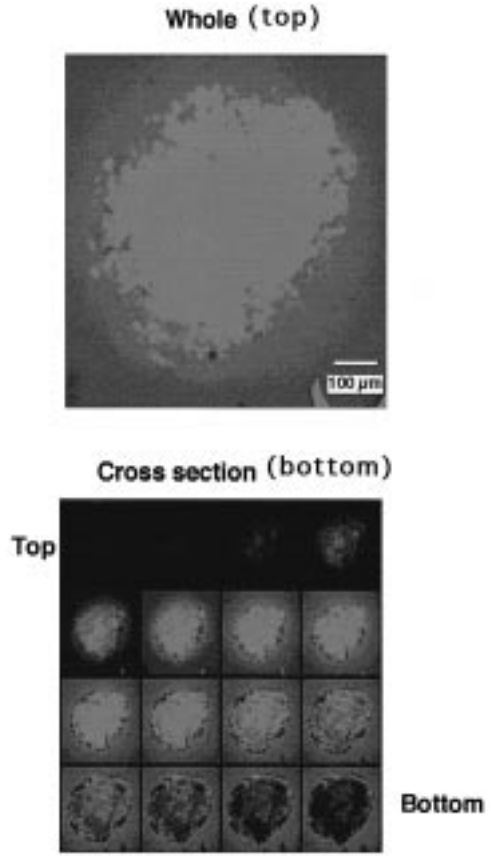

Fig. 2. Confocal micrographs of a hepatocyte aggregate stained with Alamer Blue. The whole image (top) and the cross section images from top to bottom are shown (bottom), bar = 100 μm.

1996) and it is reasonable to suppose that EGF can induce the movement of hepatocytes. In 0.3% agarose gel, hepatocyte aggregates formed more frequently than in gel of higher concentrations. Probably because of the free movement of hepatocytes in soft gel. On the contrary, when hepatocytes are cultured on 0.3% agarose gel, they form aggregate more rapidly (within 3 h) (data not shown) but also completely die within 24 h (Figure 3).

Viability and differential activity of hepatocytes in and on agarose gel culture

Next we investigated the viability of hepatocytes cultured in and on 0.3% agarose gel. The results show that the hepatocytes cultured for 21 days on 0.3% agarose gel with 10 ng EGF/ml, 100 nM insulin, and 10% FBS are yet viable (Figure 2). Whereas, hepatocytes cultured on gel died almost completely after same period (data not shown). It has been reported that hepatocytes cultured in anchorage–independent condition, such as on agarose gel undergo apoptosis (Ruoslahti & Reed 1994). However, it is shown that under appropriate concentration of gel and culture conditions, hepatocytes could escape apoptosis in anchorage–independent culture conditions even though the nature of cell death under our experimental conditions is not analyzed.

Another research group has reported that the internal hepatocytes of the aggregates are dead cells (Koide *et al.* 1989). To address this point, we stained an aggregate of 21 days by Alamer Blue and observed the inside of aggregates using confocal microscopy. As shown in Figure 2, the internal hepatocytes of the ag-

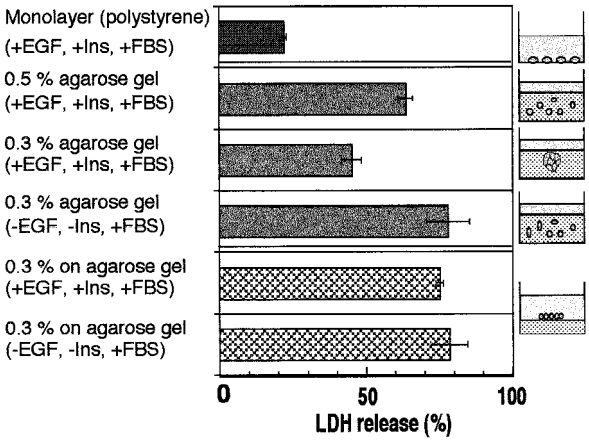

Fig. 3. Comparative effects of various culture conditions on lactate dehydrogenase (LDH) relaese in culture medium of hepatocytes. Hepatocytes (1×10^5 cells/ml) were cultured for 72 h under the indicated culture condition (Right hand). Each data point represents the group mean ± standard error (SE) ($n = 3$ per group).

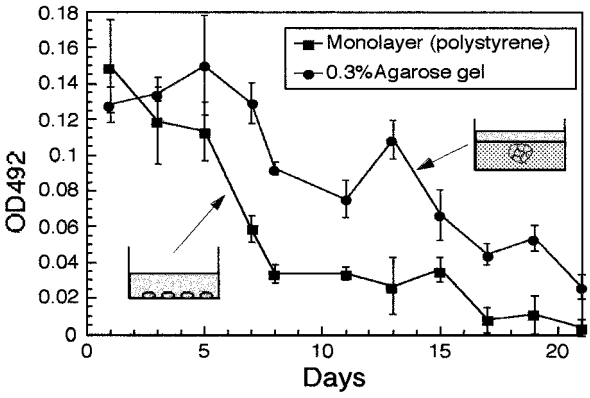

Fig. 4. Comparison of albumin concentrations at every 48 h in medium of monolayer cultured and 3D agarose gel cultured hepatocytes. The concentrations of albumin were determined by a sandwich ELISA. (●) 0.3% agarose gel and (■) monolayer cultured hepatocytes in William's E medium with 10 ng EGF/ml, 100 nM insulin, and 10% fetal bovine serum. Each data point represents the group mean ± standard error (SE) of absorbancy of *O*–phenylenediamine at 492 nm ($n = 3$ per group).

gregate are stained with the Alamer Blue, suggesting the viability of the internal hepatocytes. We, therefore, propose that the formation of hepatocyte aggregates by self–assembly under 3D culture only increase the adaptation capability of hepatocytes even in an inert surface. In fact, internal hepatocytes in aggregates are relatively viable upto 60 days and their viability is decreased with an increase in seeded cell density (data not shown). These data are consistent with our previous observation (Shinzawa *et al.* 1995).

Since LDH release in the culture medium reflects the viability of the cells under certain culture condition, for further confirmation of hepatocytes viability in 3D and 2D culture, we analyzed the LDH release in the culture medium. Figure 3 shows that the LDH release in medium of hepatocytes cultured on gel is significantly higher than in gel culture medium. These data further support the data described in Figure 2. However, high LDH release in 3D culture medium is observed if the hepatocytes are cultured without growth factors or in higher concentrations of agarose gel (Figure 3). On the other hand, slightly higher LDH release in 3D culture medium than medium from monolayer culture of hepatocytes on polystyrene dishes is most likely because of LDH release from hepatocytes that failed to form aggregates in gel.

Analysis of albumin level in culture medium after different days support the above hypothesis. Note that albumin level is higher in medium of 3D culture than that of monolayer culture (Figure 4) even after more than 20 days. Taking together, these results clearly imply that the formation of hepatocyte aggregates not only increases cell viability but also maintains their functional activity under 3D culture (Figures 2–4) but not in 2D culture conditions (Figure 3). Moreover, both gel concentration and growth factor(s) affect these parameters.

To our knowledge, this is the first report to demonstrate the comparative effects on hepatocytes viability and differential activity upon 2D and 3D culture in agarose gel. It is shown that the 3D culture in appropriate concentration of gel has much better effect than 2D culture of hepatocytes (Figures 2–4). Although the internal cells of hepatocyte aggregates were reported to be dead cells, we showed that they are alive if cultured in 0.3% gel under our culture conditions (Figure 2). Finally, they possess a high levels of albumin synthesis for more than 20 days (Figure 4), suggesting that the hepatocytes in aggregates are functionally active and deserve further attention to evaluate their possible clinical applicability as well as to study hepatocyte biology in health and diseases.

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References

Berthiaume F, Moghe PV, Toner M, Yarmush ML (1996) *FASEB* **10**: 1471–1484.

Hasen LK, Hsiao C, Friend JR, Wu F, Bridge GA, Remmel RP, Cerra FB, Hu W (1998) *Tissue Eng.* **4**: 65–74.

Lee YC, Lee RT (1994) *Neoglycoconjugates*. San Diego: Academic Press, pp. 262–282.

Koide N, Shinji N, Tanabe T, Asano K, Kawaguchi M, Sakaguchi K, Koide Y, Mori M, Tsuji T (1989) *Biochem. Biophys. Res. Commun.* **161**: 385–391.

Mooney DL, Hansen LK, Vacanti JP, Langer R, Farmer SR, Ingber DE (1992) *J. Cell. Phys.* **151**: 497–505.

Mooney DJ, Organ G, Vacanti JP, Langer R (1994) *Cell Transplant.* **3**: 203–210.

Morita M, Watanabe Y, Akaike T (1995) *Hepatology* **21**: 1585–1593.

Nishikawa Y, Tokusashi Y, Kadohama T, Nishimori H, Ogawa K (1996) *Exp. Cell. Res.* **223**: 357–71.

Ruoslahti E, Reed JC (1994) *Cell* **77**: 477–478.

Shinzawa K, Watanabe Y, Akaike T (1995) *Cell Death and Differentiation* **2**: 133–140.

Tong JZ, Bernard O, Alvarez F (1990) *Exp. Cell Res.* **87**: 87–92.

Wu F, Friend JR, Hsiao CC, Zilliox MJ, Ko WJ, Cerra FB, Hu WS (1996) *Biotechnol. Bioeng.* **50**: 404–415.