USE OF LIPOSOME ENCAPSULATED HEMOGLOBIN (LEH) AS AN OXYGEN CARRIER TO CULTURED CELLS

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1. Introduction

Oxygen transport is the most constraining parameter to engineer three-dimensional (3 D) tissues in vitro. The concentration of oxygen dissolved in the culture medium according to the Henry's law is very low (about 2×10^{-7} mol/mL under 21%-O_2 containing air), and it is not likely to satisfy the metabolic demand of a high cell density tissue such as liver (e.g., 2.5×10^{8} parenchymal cells/mL-in vivo liver tissue). Various methods have been employed to supply cells with oxygen, including gas exchanger, aeration, and gas-permeable membrane-based exchange. In addition to these conventional methods, uses of modified red blood cells has been emergent.

Another approach is the use of blood substitutes that have been developed primarily for infusion to human patients. There are two main types of oxygen carriers: hemoglobin-based oxygen carriers (HBOCs) (1) and perfluorocarbon-based oxygen carriers (PFCs) (2). Because PFCs do not exhibit an oxygen binding and dissociation curve similar to that of red blood cells, actual uses of hemoglobin (Hb) is ideal, particularly for in vitro cell culture. One of the most promising HBOCs is liposome-encapsulated Hbs (LEH). The advantages of LEH over other oxygen carriers are as follows: Purified hemoglobin (Hb) protein is encapsulated in a phospholipids bilayer membrane to escape from various trapping processes *in vivo* and to prevent the leakage of free Hb, which induces vasoconstriction and renal toxicity in mammals when it directly come into contact with living tissues; It has no blood type antigens and infectious viruses, and can be stored for long time periods (~ 2 years). Such vigorous efforts have been made to introduce LEH into final clinical trials (3).

Although necessities of oxygenation using HBOCs were widely recognized in vitro cell culture, there are almost no previous reports that checked their feasibility of engineering 3 D hepatic tissues. In this study, first, we investigated LEH's possible toxicity to cultured cells including both hepatoma Hep G2 cells and primary rat hepatocytes, when it comes into contact in a direct manner, that is, supplementation to the culture medium. We used a new type of LEH, in which the outer surfaces of the phospholipids bilayers of the capsules in a mean diameter of 269 nm are further modified with polyethyleneglycol to extend its *in vivo* residence time. Second, using a flat-plate bioreactor (4,5) to form an oxygen gradient in a small-scale culture, we investigated the effects of LEH on the morphologies and functions of rat hepatocytes. Our results first demonstrate the high feasibility of LEH uses in direct supplementation to culture medium and show a promise in engineering large-scale organ equivalents in vitro.

2. Materials and Methods

2.1 *Hepatocyte isolation and culture*

Human hepatoma Hep G2 cells were purchased from the Japanese Cancer Research Bank (JCRB). The basal culture medium was composed of DMEM supplied with 10% fetal bovine serum, 20 mM HEPES, 1% NEAA, 100 U-penicillin/mm and 100 μg-streptomycin/ml. Subcultivation of the cells was performed every 4 d with trypsin/EDTA.

Primary rat hepatocytes were isolated from 5-6 week old adult male Wistar rats' liver according to the conventional collagenase-perfusion procedures. The basal culture medium was composed of DMEM supplemented with 1% NEAA, 20 mM HEPES, 100 units penicillin/ml, 100 μg streptomycin/ml, 10^{-7} M insulin, 10^{-6} M dexamethasone, 10 ng/ml mouse epidermal growth factor, 10^{-7} M $CuSO_4$, 10^{-8} M H_2SeO_3, 10^{-6} M $ZnSO_4$, and 10^{-6} M $MnSO_4$.

2.2 Liosome-encapsulated Hbs (LEH).

LEH were obtained from Oxygenix Co. Ltd (Tokyo, Japan). The LEH were packaged in a bottle full of N_2 and stored at 4℃. We drew LEH out by a sterile needle (Terumo, Japan) and filtered them through a 0.45μm cellulose acetate filter (Adavantec, Japan).

The LEH were suspended in 0.9 % NaCl solution and the concentration of Hb and lipid were 10 g/dl and 7 g/dl, respectively. The mean diameter of LEH was 269 nm. The metHb level was 8.5%. The oxygen affinity (P50) was 23 mm Hg, similar to red blood cells (RBCs) (P50 = 26 mm Hg).

Bovine serum albumin (BSA) is expected to inhibit the toxicity of LEH. The composition of medium is summarized in Table 1.

Table 1. Culture conditions in monolayer cultures

Type	Composition
BSA (-) LEH (-)	Basal medium, 20% (v/v) NaCl*
BSA (+) LEH (-)	BSA-contained medium#, 20% (v/v) NaCl
BSA (-) LEH (+)	Basal medium, 20% (v/v) LEH
BSA (+) LEH (+)	BSA-contained medium, 20% (v/v) LEH

NaCl*: 0.9 % NaCl solution; BSA-contained medium #: 4 g/L in medium, physiological concentration

To investigate LEH's possible toxicity to cells, Hep G2 cells (5×10^4 cells/cm^2) and rat hepatocytes (1×10^5 cells/cm^2) were cultured on collagen-coated 12 well-plate (3.8 cm^2; IWAKI, Tokyo, Japan) in basal medium for 24 h, and then the medium was changed to the fresh medium (Table 1) every day. Hep G2 cells and rat hepatocytes were cultured for 6 days and 3 days, respectively, with and without supplementation of LEH to the culture medium. Spent culture media at each medium exchange were stored for functional measurement.

2.3 2D flat-plate bioreactor

To form steady-state oxygen gradients *in vitro*, we used a flat-plate bioreactor (4). A slide glass (26×76 mm) (Matsunami, Japan) precoated with 0.03% collagen and a Viton gum strip (thick 0.5 mm) were inserted to 2 transparent acrylate resin plates to construct the bioreactor (Figure 1). The bioreactor was bounded and well sealed with 12 screws. The effective culture area was 20 mm (width) ×70 mm (length) ×0.5 mm (height). The whole perfusion circuit consisted of a reservoir bottle (50 mL)

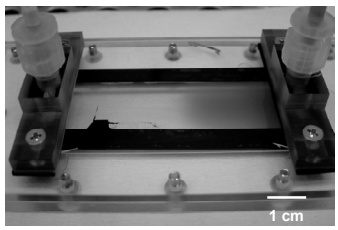

Figure 1. A flat-plate bioreactor.

with a gas-permeable silicon membrane cap, a peristaltic pump (SJ-1220; Atto Biointrustment, Tokyo, Japan), a glass bubble trap (Y.M.A. Science, Tokyo, Japan), and a flat-plate bioreactor. The bioreactor was sterilized by ethylene oxide gas (EOG).

To investigate the effects of LEH (+/-) on the cell culture under oxygen gradients *in vitro*, rat hepatocytes were cultured in the flat-plate bioreactors. Isolated primary rat hepatocytes (1×10^5 cells/cm^2) were seeded to a collagen-coated slide glass (26×76 mm) in a customer-defined rectangle bioreactor just to contain the slide glass for static culture. After 6 hours of cultivation, the medium was changed. The cells were allowed to attach to the slide-glass for another 18 hours untill the uniformly attached cells was observed. Then, the slide glasses were inserted into the flat-plate bioreactors and the cells were cultured in the medium supplied with the LEH (+/-) for 24 hours perfusion. The flow rate was 0.1 ml/min, with which the oxygen concentration at the outlet of the bioreactor without using LEH was minimized at about 3 ppm (4). As a control group, cell-loaded slide glass was cultured in the same rectangle bioreactor above without perfusion. All cultures were held under 5 % CO_2/95 % air at 37℃.

2.4 Cell morphologies and measurement of metabolic functions

All cell morphologies were observed by a phase-contrast microscope. The concentration of glucose in the culture medium was measured by a glucose analyzer (Glucose Analyze 2; Beckman Instruments, Galway, Ireland). The concentration of human and rat serum albumin produced was measured by a sandwich-type enzyme-linked immunosorbent assay (ELISA).

3. Results and Discussion

3.1. Effects of LEH (+/-) on monolayer-cultured hepatoma HepG2 cells

LEH-added culture exhibited cytotoxicities as shown in Figures 2 and 3. Cellular morphology was affected by the supplement of LEH (+/-) (Figure 2). The cells without LEH exhibited their normal morphologies and growth well during 6 day of culture (Figure 2. A and B). However, deteriorating effect was observed in the cells supplemented with LEH: The cells were

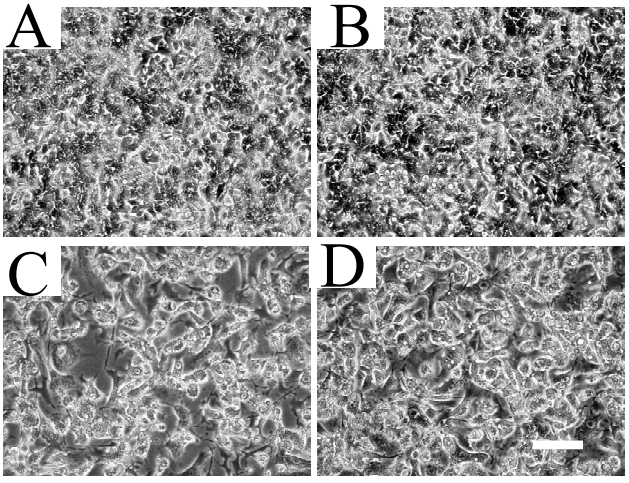

Figure 2. Morphologies of Hep G2 cells on day 6 cultured in the medium supplemented with BSA (-) LEH (-) (A), BSA (+) LEH (-) (B), BSA (-) LEH (+) (C) and BSA (+) LEH (+). Bar indicates 100 μm.

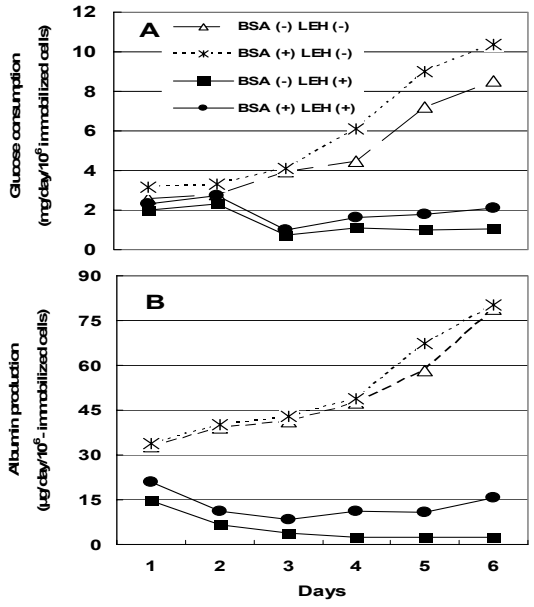

Figure 3. Glucose consumption (A) and albumin production (B) of Hep G2 cells.

distressed and detached from the culture dishes with an increase of culture time (Figure 2. C and D). There were no significant morphological differences between the cultures with and without BSA. The two functions of glucose consumption and albumin production (Figure 3) also reflected the cytotoxicity by supplementation of the LEH, although BSA supplementation slightly reduced the toxic effect.

The exact mechanism of the observed cytotoxicity was not clear. One possible explanation is as follows, most of the LEH particles might be ingested by hepatoma Hep G2 cells, which have an abnormally enhanced capacity to uptake large molecules such as lipid, and the concentration of intracellular free hemoglobin from the LEH increased, which caused cytotoxity (6).

3.2. Effects of LEH (+/-) on monolayer-cultured primary rat hepatocytes

Unlike the results on Hep G2, the two functions of cultured primary rat hepatocytes were not affected by the added LEH; both functions were well maintained throughout the 3 days of culture (Figure 4). The glucose consumption on Day 3 in LEH-added medium was slightly higher than that without LEH. There were no significant functional differences between the cultures with or without BSA. These data confirmed that the exposure of primary rat hepatocytes to high concentrations of LEH (20%) up to 3 days did not adversely affect the functions, suggesting the high feasibility of the LEH in the culture of normal hepatocytes.

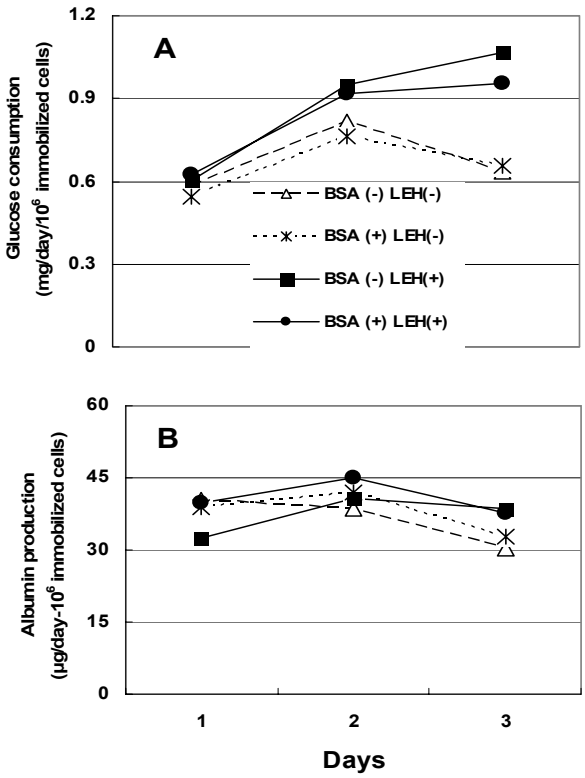

Figure 4. Glucose consumption (A) and albumin production (B) of rat hepatocytes.

3.3. Effects of LEH (+/-) on 2D perfusion-cultured primary rat hepatocytes

After confirming there are no adverse effects of LEH on rat hepatocytes, we continued to investigate the effects of LEH and oxygen gradients on cells. Figure 5 shows the results of cell morphology of rat hepatocytes cultured in 2D flat-plate bioreactors. Cellular morphology was affected by the medium composition (with or without LEH) and the region of the slide-glass. Without the LEH, the activity of the cells gradually decreased toward the downstream areas, though almost all cells in the inlet region were completely alive and maintained their good morphologies. In the outlet region, cells were dead and detached from the slide-glass. In contrast, with the LEH, all the cells maintained their good morphologies all over the region, from the inlet to the outlet. The cellular morphologies might be affected by the oxygen gradients formed in the region of the 2D slide-glass, in which the oxygen tension generally decreased toward the downstream. As reported by Allen *et al*, the oxygen level affected the activity of hepatocytes (4). In this study, we also observed that the cells exhibited different activity along the flow region having different oxygen concentrations. In addition, the improved oxygen transport by supplementation of LEH enhanced the cells' activity even in the downstream region.

Figure 6 shows the effects of the LEH (+/-) on albumin production of rat hepatocytes cultured in flat-plate perfusion bioreactors. The rate of albumin secretion in perfusion supplemented with LEH was

4-times higher than that in the culture without using LEH and 1.4-times than that in the static control. These results indicated that improved oxygenation by supplementation of LEH completely recover the deteriorated functions caused by oxygen shortage.

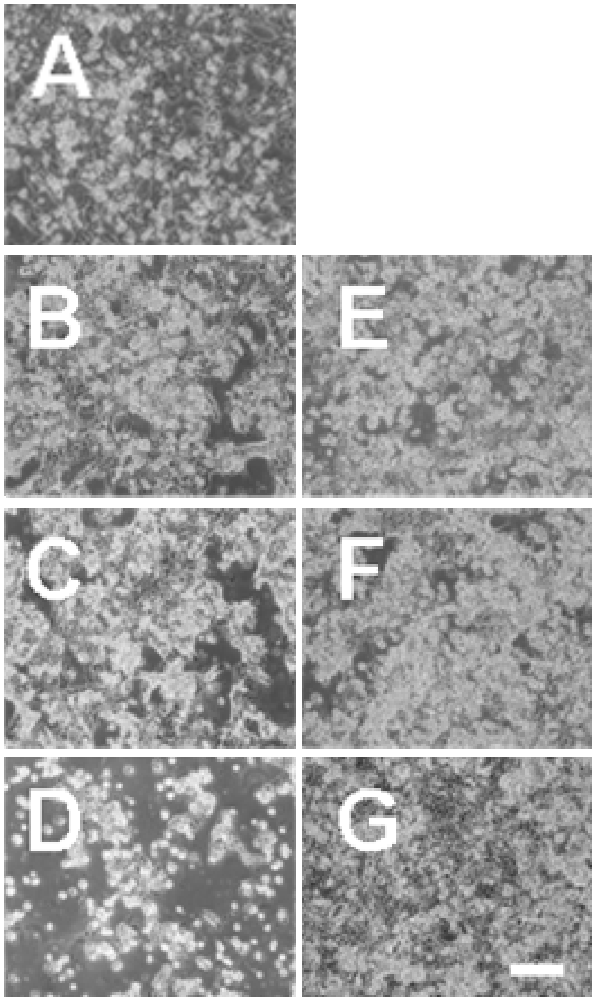

Figure 5. Morphologies of rat hepatocytes cultured in flat-plate perfusion bioreactors with and without LEH supplementation to the culture medium. (A), at 24 h immediately before the start of perfusion; (B-D), without LEH; (E-F), with LEH; (B) and (E), near the inlet; (C) and (F), in the middle; (D) and (G), near the outlet. Bar indicates 100 mm.

Albumin production by the cells in perfusion bioreactor was higher than that in the static culture. It is not clear whether perfusion itself reduced the thickness of a possible boundary layer in terms of the oxygen concentration formed over the cell layer or supplementation of LEH supplied enough oxygen to the cell layer. Clarification of the exact reason may need future research.

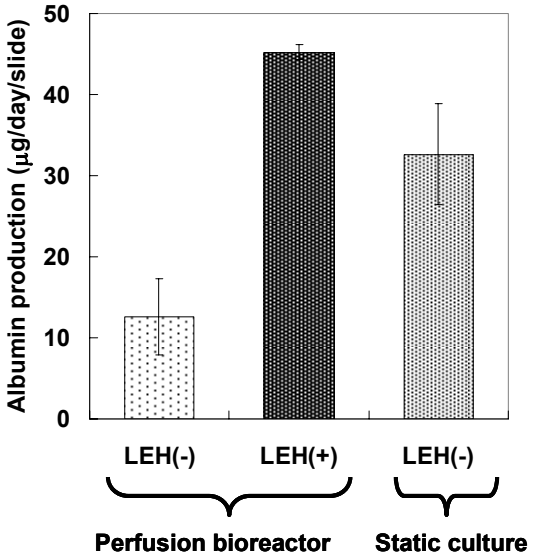

Figure 6. Albumin production of rat hepatocytes cultured in flat-plate perfusion biorectors with and without LEH supplementation to the culture medium. Data from static culture of the glass slide without LEH(-) was also shown as a control. Bar indicates maximal errors in two independent experiments.

4. Conclusion

First, we investigated the LEH's possible toxicity to hepatoma Hep G2 cells and primary rat hepatocytes. Cytotoxicities to Hep G2 during 6 days of culture were observed when supplemented with high concentrations of LEH (20%, v/v). Simultaneous supplementation of bovine serum albumin to the culture medium slightly inhibited this toxic effect. In contrast, the exposure of normal primary rat

hepatocytes to LEH in static monolayer culture up to 4 days did not adversely affect their functions and viability. Second, we examined the favorable effect of LEH on rat hepatocytes in a 2D flat-plate bioreactor where oxygen gradient can be formed in a small scale. Improved oxygenation by the supplementation of LEH successfully recovered the deteriorating cells cultured in the downstream areas where oxygen is in shortage. These results demonstrate the LEH's high feasibility in engineering organ equivalents having a large-size and a high cell density.

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