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Composition of culture medium is more important than co-culture with hepatic non-parenchymal cells in albumin production activity of primary rat hepatocytes, and the effect was enhanced by hepatocytes spheroid culture in collagen gel

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ABSTRACT

Co-culture of primary rat hepatocytes with hepatic non-parenchymal cells or sinusoidal endothelial cells for albumin production activity as an index of liver-specific function was studied. The co-cultures were effective for the expression and maintenance of albumin production activity. However, the co-culture effect was not observed when we used a suitable culture medium, which had already been reported to be sufficient for albumin production activity. Albumin production of dispersed cells in collagen gel culture was higher than that of spheroid culture. In addition, albumin production of spheroids in collagen gel culture was higher than that of spheroid culture and dispersed cell collagen gel culture with a suitable culture medium. We found that culture medium composition was more important than co-culture for expression and maintenance of albumin production. Furthermore, we found that cell–cell interaction was effective for the expression of albumin production, but heterotypic cell–cell interaction was not necessary.

1. Introduction

Development of a practical artificial organ and a tissue-engineering technology is one of the most important fields in biochemical engineering. Especially, liver is the central internal organ of metabolism in our body, but the functional maintenance of hepatocytes is very difficult in vitro culture [1]. Therefore, creating a functional culture method for primary hepatocytes is important for developing liver tissue engineering and hybrid artificial liver. Hepatocyte organoid culture methods, represented by spheroids [1], functional culture medium [2,3], co-culture [2,4,5] and functional culture substratum [6], have been developed successfully, and they have enabled culture of hepatocytes that maintain liver-specific functions. In particular, co-culture has been focused upon because of the similarity of the microenvironment around hepatocytes in the living body [4].

Co-culture leads to functional improvement of hepatocytes, and soluble factors derived from co-cultured cells [4,5] and cell–cell interaction between heterotypic cells [7] have been reported. On the other hand, expression of liver function is low in conventional culture medium, and various factors act against liver function expression [5,8]. Co-culture effects that lead to high expression and maintenance of liver functions are desirable when we consider applications such as liver tissue engineering and artificial liver development. However, it is necessary to examine the co-culture effect in a culture system in which high expression and maintenance of liver functions are possible, when we consider applications such as liver tissue engineering and artificial liver development.

In this study, we examined the co-culture effect of hepatocytes with hepatic non-parenchymal cells (NPCs) by using collagen gel [9] and spheroid [10] culture methods, which are well known as effective methods for the expression of liver-specific functions in primary hepatocytes.

2. Materials and methods

2.1. Cells and culture media

Primary rat hepatocytes and hepatic NPCs were isolated from 7- to 9-week-old male Wistar rats (Kyudo, Tosu, Japan). Hepatocytes were prepared using a two-step collagenase perfusion method [11], and their viability was confirmed to be >85% using Trypan blue exclusion. Sinusoidal endothelial cells (SECs) were isolated from the non-parenchymal fraction mentioned above using a two-step Percoll gradient method (25/50%) [12], and their viability was 83 ± 7%.

We used five types of culture medium.

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(1) DMEM: Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD, USA).
(2) D-HDM: DMEM supplemented with 50 μg/L epidermal growth factor (EGF; Biomedical Technologies, Stoughton, MA, USA), 10 mg/L insulin from bovine pancreas (Sigma), 7.5 mg/L hydrocortisone (Sigma) and 60 mg/L L-proline (Sigma). The other supplements in the medium were the same as those previously described [13].
(3) Hu-M: Humedia EB2 supplemented with 10 μg/L human EGF, 5 μg/L human fibroblast growth factor (FGF)-β, 1 mg/L hydrocortisone, 10 mg/L heparin and 2% FBS. This medium was obtained from Kurabo (Osaka, Japan).
(4) D-HDM + VEGF: D-HDM supplemented with 10 μg/L vascular endothelial growth factor (VEGF; R&D Systems Inc., Minneapolis, MN, USA).
(5) D-HDM + Hu-M: this medium consisted of 50% D-HDM and 50% Hu-M. All supplements of D-HDM and Hu-M were added in this medium at the same final concentration. 50 μg/L EGF and 7.5 mg/L hydrocortisone were used in this study.

This experiment was reviewed by the Ethics Committee on Animal Experiments of Kyushu University.

2.2. Cell culture

The isolated cells were premixed and inoculated. We used Cellmatrix Type I-A (Nitta Gelatin, Osaka, Japan) for collagen gel culture. The collagen concentration in gel culture was 2.4 mg/mL. Cell suspensions with predetermined density were prepared in ice-cold collagen solution, and 0.1 mL of this was poured in each well of a 48-well plate. The plate was incubated for 30 min for gelation. Then, 0.5 mL medium was added onto the gel. The plate was cultured on a rotary shaker at 60 rpm. Medium was changed at 4 and 24 h after inoculation and every 48 h thereafter. We used low-cell-binding 96-well round-bottomed plates (Sumilon Celltight Spheroid; Sumitomo Bakelite Co., Tokyo, Japan; MPC polymer-coated plate; Nunc, Tokyo, Japan) for spheroid culture. 0.2 mL cell suspension was inoculated into the 96-well plates, and then it was cultured on a rotary shaker at 60 rpm. Medium was changed 24 h after inoculation and every 48 h thereafter. Spheroids in collagen gel culture were also performed. Spheroids were formed in MPC polymer-coated 48-well plates for 2 days. Then, the spheroids were collected and used for collagen gel culture. Each culture condition was investigated using three independent wells except *p*-acetamidophenol metabolism (two independent wells).

2.3. Hepatocyte function

Hepatocyte function was evaluated by examining the albumin production activity. The concentration of albumin in the culture medium was measured by an ELISA method using a protein detector ELISA kit HRP/ABTS system (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) [14]. A rat albumin standard and anti-rat albumin antibody were purchased from ICN Pharmaceuticals (Aurora, OH, USA). In the case of collagen gel culture, we calculated the net albumin production by subtracting the remaining albumin level in collagen gel, by using the hypothesis that the albumin concentrations in the medium and gel were the same. Albumin production activity per unit cell number was estimated using the inoculum cell number in this study. In addition, drug metabolism activity was evaluated by using 1.0 mM *p*-acetamidophenol containing culture medium (Wako Pure Chemical, Osaka, Japan) in some culture conditions. *p*-Acetamidophenol was used as a model drug of phase II reaction of drug metabolism, and the concentration in the culture medium was measured using a HPLC system (Waters, Milford, MA, USA) according to the previous paper [15].

2.4. Statistical analysis

The results are given as means ± SD. Statistical analysis was performed using a two-tailed unpaired Student's *t*-test (Figs. 3A, B and 5A) or a multiple comparison with Dunnett's test (Figs. 1, 2A, B, 4A, B, 5B and Table 1). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. DMEM

There have been reports about the co-culture effect with hepatocytes and NPCs in FBS-supplemented medium [16,17]. Therefore, DMEM was used for dispersed co-culture in collagen gel with primary rat hepatocytes and hepatic NPCs. There was no co-culture effect a hepatocyte:NPC ratio of 1:1, but expression and maintenance of albumin production was effective at a ratio of 1:5 and 1:10 (Fig. 1). However, the albumin production per number of hepatocytes (Fig. 1) was less than one-tenth of that in hepatocytes cultured in D-HDM (50–100 μg/10^6 cells/day [2]). Therefore, we decided to investigate the co-culture effect with D-HDM.

3.2. D-HDM

In dispersed collagen gel culture, hepatocytes in D-HDM expressed about 20 times higher albumin production than that in DMEM (Fig. 2A). The albumin production rate of hepatocytes was increased in the presence of NPCs after 11 days of co-culture, but this effect may have been caused by inhibition of the tendency to decrease albumin production, because it decreased with time under all conditions during this period (Fig. 2A). On the other hand, the co-culture effect of hepatocytes with NPCs after day 9 in spheroid culture was more remarkable than that in collagen gel culture, and 51% of albumin production activity at day 1 was maintained at day 23 in spheroid culture of hepatocytes and NPCs at a ratio of 1:10 (Fig. 2B). However, the albumin production rate per unit number of hepatocytes in the spheroid culture was about a quarter of the rate

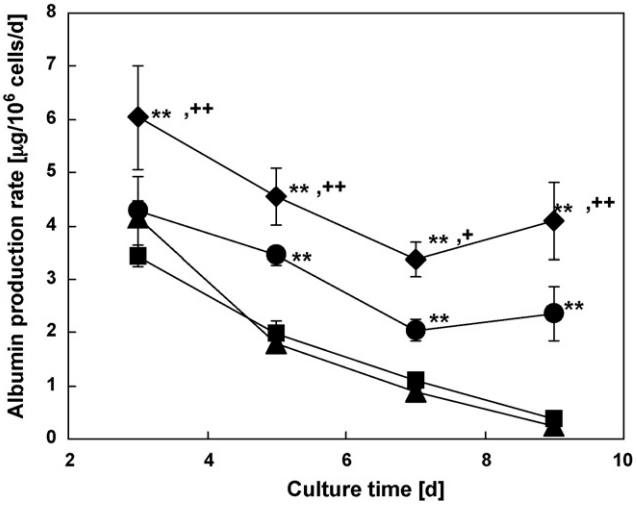

Fig. 1. Albumin production of hepatocytes cultured in DMEM. Hepatocytes were co-cultured with hepatic NPCs in collagen gel. There were 1×10^5 cells/well in 48-well plates. Ratio of hepatocytes to NPCs at inoculation was: 1:0 (triangles); 1:1 (squares); 1:5 (circles); and 1:10 (diamonds). *, +: statistically significant differences from hepatocyte:NPC ratios of 1:0 and 1:5, respectively. **, ++: $P < 0.01$; *, +: $P < 0.05$.