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Gene expression of liver-specific proteins in hepatocyte spheroids in primary culture.

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Abstract

Adult rat hepatocytes assemble to form multicellular spheroids under non-adherent environments such as immobilized chondroitin sulfate-proteoglycan in primary culture. Previously, we demonstrated that hepatocyte spheroids exhibited various differentiated structures as observed in the liver tissue. It was also shown that hepatocyte growth was highly suppressed and several differentiated functions, including albumin production and gluconeogenesis, were well preserved in spheroids. To investigate the differentiated functions of cultured hepatocytes in relation to cell morphology, we compared the expression of the albumin and transferrin genes in spheroids with those in monolayers by Northern blot analysis. Production of these proteins in the culture medium was simultaneously examined by ELISA. Gene expression and protein production of both albumin and transferrin were better preserved in spheroids. We also examined changes in the expression of liver-specific genes in response to IL-6. Reduced mRNA levels of both albumin and transferrin was only found in spheroids and no change was observed in monolayers. These results suggest that the regulation of tissue-specific gene expression is better preserved in spheroids, in which hepatocytes are in close contact with each other.

KEYWORDS: hepatocyte, spheroid, primary culture, gene expression, IL-6

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Gene Expression of Liver-Specific Proteins in Hepatocyte Spheroids in Primary Culture

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Adult rat hepatocytes assemble to form multicellular spheroids under non-adherent environments such as immobilized chondroitin sulfate-proteoglycan in primary culture. Previously, we demonstrated that hepatocyte spheroids exhibited various differentiated structures as observed in the liver tissue. It was also shown that hepatocyte growth was highly suppressed and several differentiated functions, including albumin production and gluconeogenesis, were well preserved in spheroids. To investigate the differentiated functions of cultured hepatocytes in relation to cell morphology, we compared the expression of the albumin and transferrin genes in spheroids with those in monolayers by Northern blot analysis. Production of these proteins in the culture medium was simultaneously examined by ELISA. Gene expression and protein production of both albumin and transferrin were better preserved in spheroids. We also examined changes in the expression of liver-specific genes in response to IL-6. Reduced mRNA levels of both albumin and transferrin was only found in spheroids and no change was observed in monolayers. These results suggest that the regulation of tissue-specific gene expression is better preserved in spheroids, in which hepatocytes are in close contact with each other.

Key words: hepatocyte, spheroid, primary culture, gene expression, IL-6

Liver has various synthetic and metabolic functions including production of liver-specific proteins, gluconeogenesis, glycogen metabolism, urea synthesis, synthesis and secretion of bile juice, and detoxification. Liver tissue consists of hepatocytes, some kinds of non-parenchymal cells, and extracellular matrix surrounding the hepatocytes. Recently, various adhesion molecules involved in cell-matrix and cell-cell interactions have been discovered, and their localization and functions have been intensively investigated. Cell growth and differentiation are regulated by both cell-matrix and cell-cell interactions which cause cytoskeletal changes or which induce intracellular signal transduction systems (1).

In primary culture of adult rat hepatocytes, different culture substrata induce different cell morphology. When hepatocytes are cultured in dishes coated with type I, III, and IV collagen, laminin, and fibronectin, they assemble to form sheet monolayers (hepatocyte monolayers) (2-4). However, owing to its morphology, the hepatocyte monolayer is unlikely to allow strong cell-cell interactions between hepatocytes, such as those present *in vivo*. On the other hand, as we reported in previous studies, hepatocytes assemble to form multicellular spheroids in non-adherent environments such as on dishes coated with liver-derived dermatan sulfate proteoglycans (5) or those with positively charged surfaces (6). It has been reported by others that spheroids are also formed on dishes coated with poly-HEMA (2-hydroxyethyl methacrylate) (7) and on polyurethane (8). We have also demonstrated that hepatocyte spheroids exhibit various differentiated structures as observed in the liver tissue *in vivo*. For example, junctional complexes and bile canaliculi-like structures could be observed in spheroids in which hepatocytes were in close contact with each other (6). It has also been shown that the growth of hepatocytes is highly suppressed in spheroids, leading to better preservation of various differentiated functions including albumin production (9, 10). Although transferrin and albumin are two of the major liver-specific proteins, transferrin has not been examined with cultured hepatocytes to the extent that

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albumin has.

Interleukin-6 (IL-6) is one of the major regulators of liver-specific proteins. In an *in vivo* study in rats, the administration of recombinant human IL-6 leads to a decrease in albumin mRNA levels (11). However, the response of hepatocytes to such cytokines *in vitro* has not been well investigated.

In this study, to investigate the differentiated functions of primary cultured hepatocytes in relation to cell morphology, we compared the gene expression of the liver-specific proteins, albumin and transferrin, in spheroids with those in monolayers. To determine whether there were differences in transcriptional patterns between these two genes, we examined not only albumin but also transferrin. We also examined the changes in gene expression of albumin and transferrin induced *in vitro* by stimulation with IL-6.

Materials and Methods

Culture dishes. Polystyrene culture dishes 100 mm in diameter (Primaria, Falcon, Becton Dickinson Labware, USA) were used. For spheroid culture, the dishes were either simply rinsed with phosphate-buffered saline (PBS) just before receiving cells as described previously (6) or coated with 40 μg per dish of a synthetic chondroitin sulfate proteoglycan analog (CS-PE) (Seikagaku Kogyou Co., Ltd., Japan) in which chondroitin sulfate was chemically conjugated with phosphatidylethanolamine at the reducing end (12). For monolayer culture, each dish was coated with 200 μg of type I collagen from porcine skin (Cellmatrix type IA, Nitta Gelatin, Japan), air-dried, and sterilized by UV irradiation for 16 h.

Culture. Hepatocytes were isolated from male Sprague-Dawley rats (200–270 g) by a liver perfusion method with 0.05 % collagenase, under oxygen-ether anesthesia (13). More than 80 % of the isolated cells were viable as measured by the trypan blue dye exclusion test. The medium used was serum-free hormonally defined medium (HDM) (14), comprised of Williams #E medium supplemented with 10 μg/ml insulin (Sigma Chemical Co., Mo, USA), 0.1 μM copper ($CuSO_4 \cdot 5H_2O$), 50 pM zinc ($ZnSO_4 \cdot 7H_2O$), 3 nM selenium (H_2SeO_3), 20 ng/ml mouse epidermal growth factor (Takara Shuzo Co., Ltd., Japan), 50 μg/ml linoleic acid (Sigma), 20 mU/ml prolactin, 10 μU/ml somatotropin, 100 U/ml penicillin, and 100 U/ml streptomycin. The hepatocytes were inoculated at a density of 3×10^6 cells per dish containing 10 ml of medium and cultured under a humidified atmosphere of 5 % CO_2 and 95 % air at 37°C. The medium was fully changed at 1, 3, and 5 days of culture, unless otherwise specified. In spheroid culture, the medium in which spheroids were floating, was aspirated off and centrifuged at 500 rpm for 1 min. The collected spheroids were resuspended in 10 ml of fresh HDM and seeded again in the same dish to continue the culture. In monolayer culture, medium was changed by simply aspirating off spent medium and adding fresh HDM. The medium and cells were collected separately at 1, 3, 5, and 7 days of culture and stored at −80°C until use.

Analysis of protein production. Albumin secreted into the medium was analyzed by enzyme-linked immunosorbent assay (ELISA) using peroxidase-conjugated rabbit IgG fraction specific to rat albumin (Cappel Ourham, NC, USA) with rat albumin (Cappel) as a standard. Transferrin secreted into the medium was also measured by ELISA using the rabbit IgG fraction specific to rat transferrin (Cappel) as the 1st antibody, peroxidase-conjugated anti-rabbit immunoglobulin as the 2nd antibody, and rat transferrin (Cappel) as a standard.

Measurement of DNA. Cellular DNA of the cultured hepatocytes used for the experiment of protein production was quantified. DNA was extracted using a SepaGene kit (Sankojunyaku Co., Ltd., Japan) and measured fluorometrically using a TKO 100 mini-fluorometer (Hoffer Scientific Instruments, USA) (15).

Analysis of gene expression. Total cellular RNA was extracted from cultured hepatocytes by the acid guanidinium-phenol-chloroform (AGPC) method (16). Fifteen micrograms of RNA was loaded in each lane on 1.2 % agarose formaldehyde gels ($8 \times 10 \times 0.6$ cm) and electrophoresed at 100 V for 1.5 h. After electrophoresis, RNA was transferred onto nylon membranes (NYTRAN, Schleicher and Schuell, Germany) which were then baked at 80°C for 3 h.

Prehybridization was carried out in 20 ml of $6 \times$ SSC (SSC; 0.15 M NaCl, 0.015 M sodium citrate), $5 \times$ Denhardt's Solution (Denhardt's Solution: 0.02 % BSA, 0.02 % Ficoll 400, 0.02 % polyvinylpyrrolidone), and 0.1 % SDS at 68°C for 3 h. Hybridization was performed at 68°C overnight in 5 ml of the same solution as that used for prehybridization except for the inclusion of ^{32}P-labeled rat albumin, rat transferrin, or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH; as an internal control) cDNA probe, and 0.1 mg/ml of yeast tRNA. After

a brief rinse in 2 × SSC containing 0.1 % SDS at room temperature for 10 min twice. Then the nylon membranes were twice washed in 0.1 × SSC containing 0.1 % SDS at 68°C for 1 h, and exposed to Kodak XAR5 film at − 80°C for 1–7 days. Hybridization signals were analyzed using a laser scanning densitometer (Molecular Dynamics, model 300 SX) and analyzed semiquantitatively by calculating the relative ratio to the signal of GAPDH.

IL-6 treatment. On the 3rd day of culture, when all of the inoculated cells assembled into floating spheroids in spheroid culture and formed confluent monolayers in monolayer culture, the spent medium was replaced with fresh medium containing 13.5 ng/ml of recombinant murine IL-6 (GIBCO BRL, Grand Island, NY, USA) with 3 % BSA as a carrier protein, and culture was continued in the presence of IL-6 for 3, 6, or 12 h. After treatment with IL-6, the cultured hepatocytes were harvested and stored at − 80°C until Northern blot analysis. Changes in mRNA expression induced by IL-6 treatment were analyzed in the same manner as described above.

Results

Spheroid Formation and Tissue-specific Protein Production

Freshly isolated adult rat hepatocytes formed multicellular spheroids within the initial 3 days in culture both in non-coated Primaria dishes as previously described (6) and in dishes coated with CS-PE (Fig. 1). There was no remarkable difference in the phase-contrast microscopic

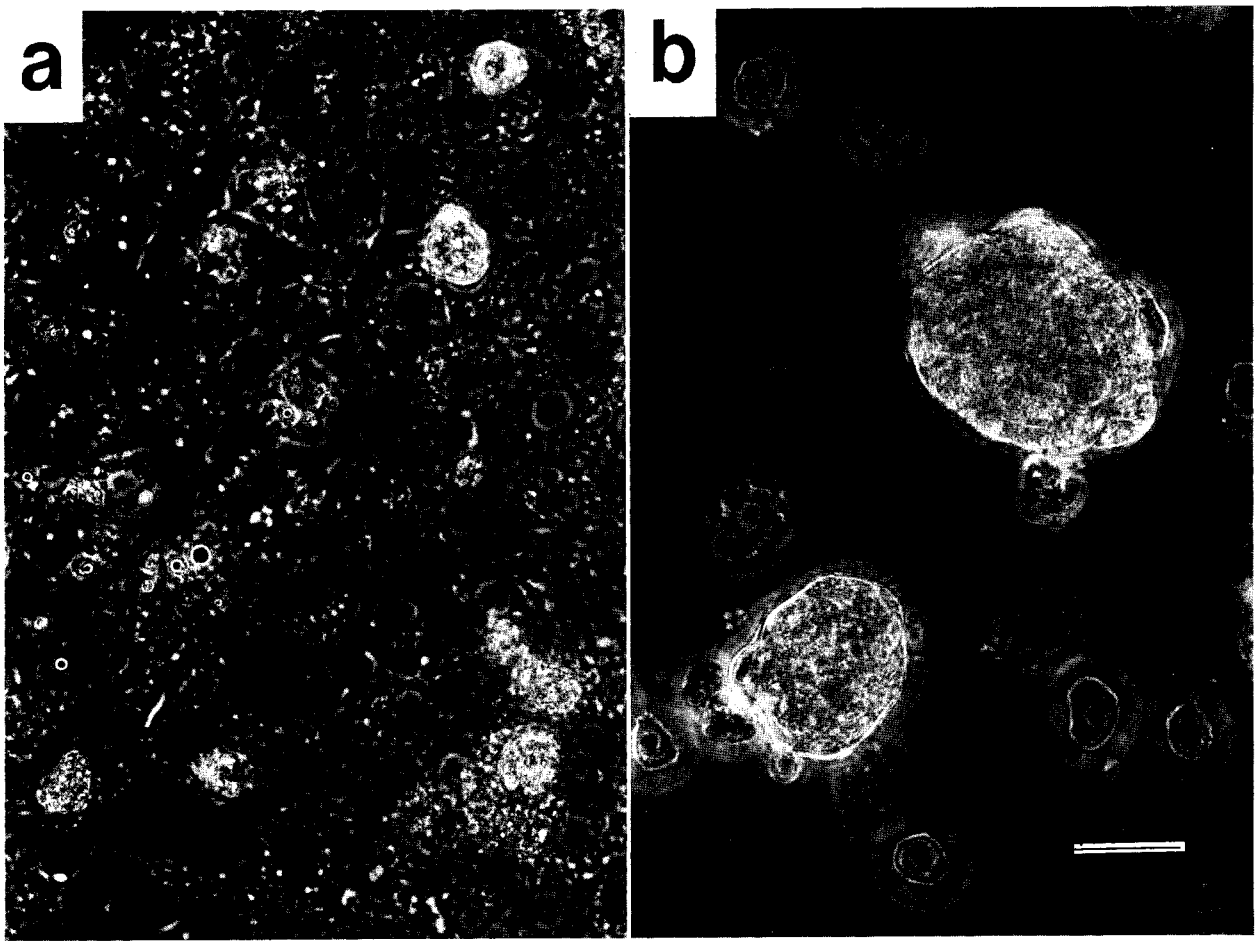

Fig. 1 Phase-contrast microscopic features of hepatocyte monolayers and spheroids. Photographs were taken 3 days after the initiation of culture. **a**, Monolayer; **b**, Spheroid; Bar = 50 μm.

appearance of spheroids between non-coated and CS-PE-coated dishes, except that the average diameter of spheroids in the latter was slightly smaller ($100 \pm 23 \mu m$) than that in the former ($110 \pm 21 \mu m$). The isolated hepatocytes seeded in type I collagen-coated dishes assembled to form confluent monolayers within the initial 3 days (Fig. 1).

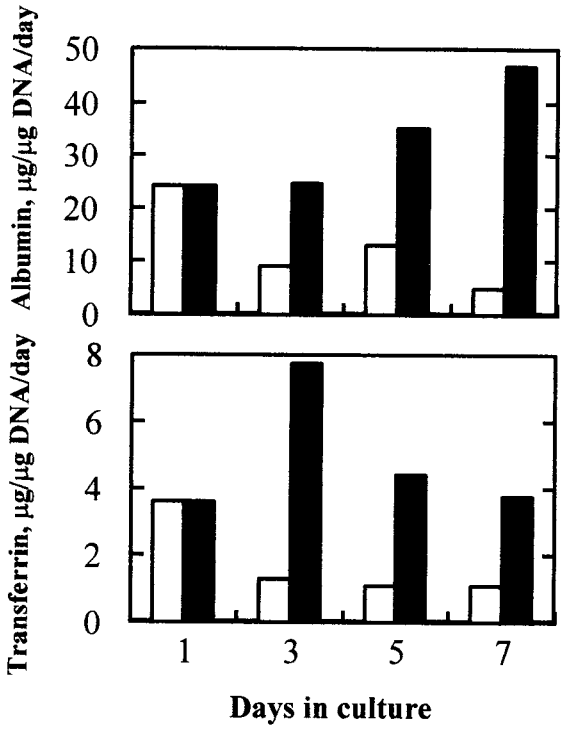

Fig. 2 Amounts of proteins secreted into the medium. The concentrations of albumin (upper column) and transferrin (lower column) in the medium collected every 2 days from monolayers (open bars) and spheroids (closed bars) were measured by ELISA.

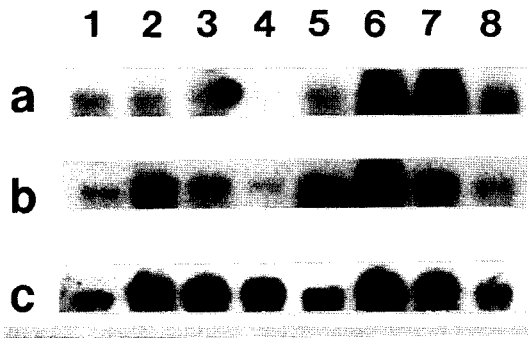

Fig. 3 Northern blot analysis of albumin and transferrin mRNA. Total cellular RNA was extracted from monolayers (lanes 1-4) and spheroids (lanes 5-8) on day 1 (lanes 1 and 5), day 3 (lanes 2 and 6), day 5 (lanes 3 and 7), and day 7 (lanes 4 and 8). The extracted RNA was electrophoresed, blotted, and hybridized to albumin (**a**), transferrin (**b**), and GAPDH (**c**) cDNA probes.

Albumin and transferrin are serum proteins produced solely by hepatocytes. To evaluate tissue-specific gene expression in the two culture systems, the secretion of albumin and transferrin per unit of cells into the medium were compared. As shown in Fig. 2, the amount of albumin in spheroid culture gradually increased after day 3, when spheroid formation was completed, while that of transferrin increased on day 3 and decreased thereafter to the initial level. On the other hand, the secretion of both albumin and transferrin in the monolayer culture were both highest on day 1 and decreased gradually thereafter until the end of culture.

Albumin and Transferrin Gene Expression

Levels of albumin and transferrin mRNAs were analyzed by Northern blot analysis. The albumin and transferrin cDNA probes detected 1.9-kb and 2.6-kb

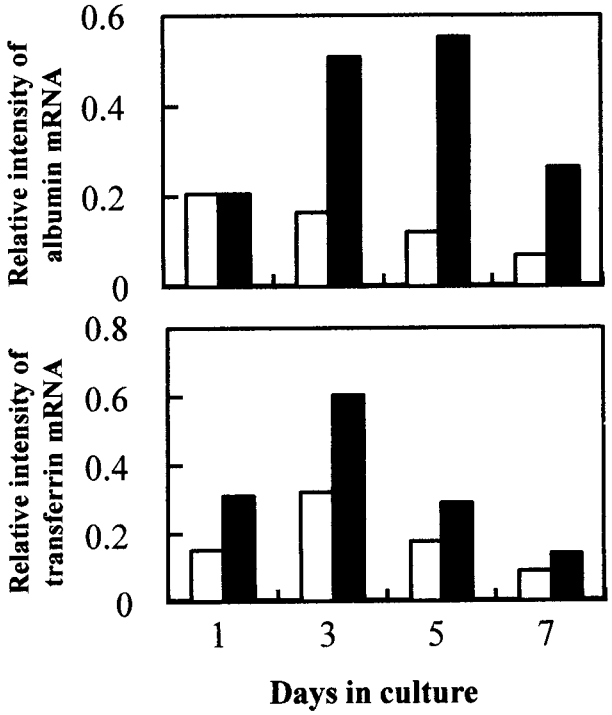

Fig. 4 Signal intensities of albumin and transferrin mRNA. mRNA signals of albumin (upper column) and transferrin (lower column) were measured by laser scanning densitometry and normalized with GAPDH mRNA signal. Open bars, monolayer; Closed bars, spheroid.

mRNA bands corresponding to rat albumin mRNA and rat transferrin mRNA (17), respectively (Fig. 3). These mRNA levels were quantified by laser scanning densitometry and normalized to that of GAPDH mRNA used as an internal control. In hepatocyte monolayers, albumin mRNA levels decreased gradually with time in culture. On the other hand, albumin mRNA levels of hepatocyte spheroids increased until the 5th day of culture, and began to decline at the 7th day (Fig. 4). In comparison with hepatocyte monolayers, culturing hepatocytes on CS-PE increased albumin mRNA three- to fourfold after the 3rd day of culture, when hepatocytes had already formed multicellular spheroids. The transferrin mRNA level peaked on the 3rd day and then declined with culture time both in hepatocyte monolayers and hepatocyte spheroids. In hepatocyte spheroids, transferrin mRNA was induced more than in hepatocyte monolayers throughout the culture period, but these levels were within twice the levels in the hepatocyte monolayer.

Modulation of Albumin and Transferrin Gene Expression by IL-6

The levels of albumin and transferrin mRNAs exhibited no significant changes in response to IL-6 treatment in the present monolayer culture system. In contrast, in spheroid cultures, the albumin mRNA level decreased slightly from 6h to 12h after administration of IL-6. The decrease in the level of transferrin mRNA in spheroid culture was more evident from 6h to 12h (Figs. 5 and 6).

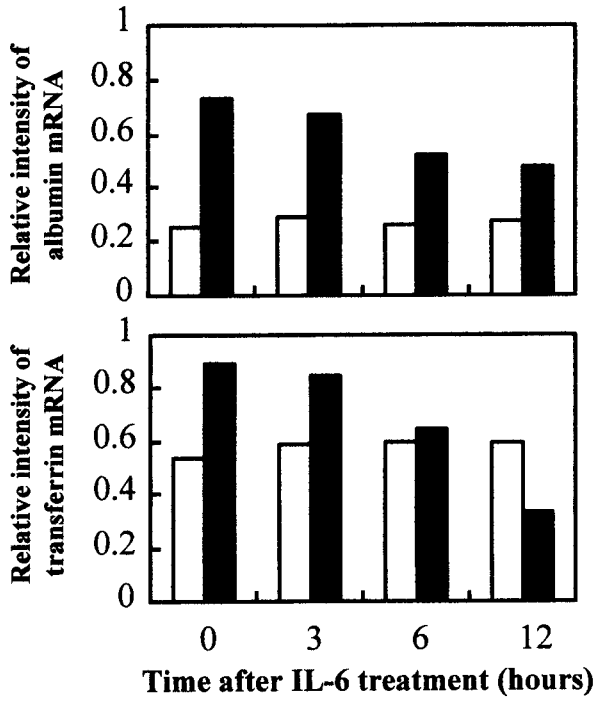

Fig. 6 Changes in signal intensities of albumin and transferrin mRNA on IL-6 treatment. mRNA signals of albumin (upper column) and transferrin (lower column) were measured by laser scanning densitometry and normalized with GAPDH mRNA signal. Open bars, monolayer; Closed bars, spheroid.

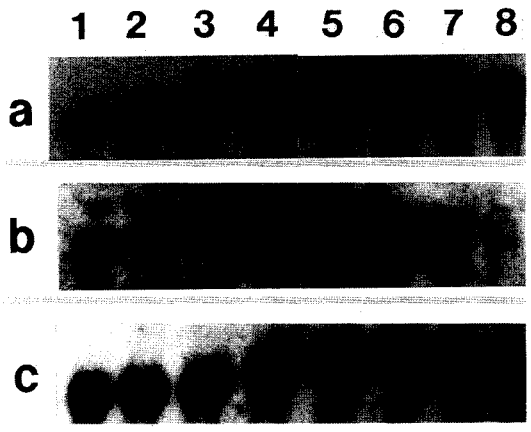

Fig. 5 Northern blot analysis of albumin and transferrin mRNA on IL-6 treatment. Total cellular RNA was extracted from monolayers (lanes 1–4) and spheroids (lanes 5–8) before treatment (lanes 1 and 5), and 3h (lanes 2 and 6), 6h (lanes 3 and 7), and 12h (lanes 4 and 8) after treatment with IL-6. The extracted RNA was hybridized to albumin (**a**), transferrin (**b**), and GAPDH (**c**) cDNA probes, respectively.

Discussion

Hepatocyte spheroids have a high capacity to produce liver-specific proteins. However, the mechanism of this production has not been examined. In this study, we compared the expression of albumin and transferrin genes in hepatocyte spheroids with that in hepatocyte monolayers. Both albumin and transferrin gene expression and protein production were better preserved in hepatocyte spheroids, in which cells made close contact than in hepatocyte monolayers. These findings suggest that the expression of tissue-specific genes is better preserved at the higher cell density achieved in the three-dimensional spheroid structure.

In hepatocyte spheroids, the secretion of albumin into the medium increased until the 7th day of culture.

However, in monolayer culture, albumin mRNA levels increased until the 5th day of culture and began to decline at the 7th day. This discrepancy between the levels of protein and mRNA can be explained if the albumin production at the 7th day actually reflects the amount of albumin secreted in the previous 2 days. The observed higher production of albumin on the 7th day than on the 5th day is considered to result from the very high level of mRNA on the 5th day.

Our results suggest that differentiated gene expression is regulated by cell density and morphology. However, the mechanism of this regulation in hepatocyte spheroids is still unknown. Recently, proteins likely participating in hepatocyte-specific transcription have been identified. For example, hepatocyte nuclear factor (HNF)-1 interacts with the albumin promoter and regulates transcription of the albumin gene in cooperation with other transcription factors (18-21). By culturing the cells on collagen gel, they become cuboidal in shape and hepatocyte nuclear factor 3α mRNA levels are strongly increased (22). HNF-3 binds to its binding site in the albumin enhancer and can mediate enhancer activation in cooperation with other transcription factors. The enhancer activation causes transcriptional activation of the albumin gene (22-24). Therefore, in hepatocyte spheroids, it is likely that some kinds of transcription factors play an important role in transcriptional regulation of liver-specific proteins. The stimulation by intercellular adhesion molecules such as E-cadherin and/or the cytoskeletal changes (25) occurring in spheroid culture activate the transcriptional factors of differentiated genes. The behavior of transcriptional factors and adhesion molecules in hepatocyte spheroids must be further investigated.

In this study, transferrin mRNA levels in hepatocyte spheroids were within twice the levels found in hepatocyte monolayers. This behavior was slightly different from that of albumin mRNA. This difference at the transcriptional level cannot be explained at present. The results of a recent study revealed that HNF-3, HNF-4, and other transcriptional factors cooperatively regulated transcription of the transferrin gene (26, 27). Similar transcriptional factors may function in transcription of albumin and transferrin genes, but the intracellular signal transduction systems for these genes might be different.

Andus *et al.* reported that the incubation of hepatocytes with IL-6 led to a 30% reduction of albumin synthesis in rat hepatocyte primary monolayer culture (28). Tong *et al.* reported that albumin synthesis in adult rat hepatocyte spheroids was only slightly decreased in the presence of IL-6 (7). However, these studies did not compare the cytokine response in monolayer culture with that in cultures of aggregates such as in spheroids under the same conditions. For example, the experiments cited above were carried out using different culture media; serum-containing medium was used for monolayer culture and serum-free medium was used for aggregate culture. In this study, we compared the responses to IL-6 treatment of hepatocyte spheroids with those of hepatocyte monolayers under the same culture conditions. Considerable decreases in the mRNA levels of albumin and transferrin were observed only in spheroids. However, the mechanism of these decreases is still unknown. IL-6 receptors and the intracellular signal transduction system with IL-6 should be examined in spheroids. Our results suggest that the regulation of liver-specific genes by IL-6 was better preserved in the spheroid culture system than in monolayers. With regard to the response to this cytokine, hepatocyte spheroids seemed to be similar to hepatocytes in liver tissue *in vivo*.

This study indicates that the regulation of tissue-specific gene expression is well conserved in multicellular spheroids, in which hepatocytes are in close contact with each other.

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