Adult rat hepatocytes cultured on an oxygen-permeable film increases the activity of albumin secretion

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Abstract

Primary culture of rat hepatocyte was performed in an oxygen-permeable film dish (F-dish), which would be expected to give an oxygen-rich culture condition. In the conventional culture dish in which the depth of medium was 2 mm, the oxygen tension (pO_2) in the medium decreased from 19% (144 mmHg) to 0.3% (2.3 mmHg) within 2 hr, while the pO_2 in the F-dish maintained 8.5% (64.6 mmHg) even after 2 hr. The adverse effect of the oxygen-deficiency appeared in the albumin secretion activity of the hepatocytes and it was more remarkable in the early period of culture. The average rate of albumin secretion for the initial 48 hr was $2.0 \mu\text{g ml}^{-1} \text{hr}^{-1}$ or $96 \mu\text{g } 10^6 \text{ cells}^{-1} \text{ day}^{-1}$ in the F-dish. The average rate of albumin secretion for the initial 12 hr was only $0.36 \mu\text{g ml}^{-1} \text{hr}^{-1}$ in the conventional culture dish. The activity of ammonia elimination in the F-dish was 20–50% higher than the conventional culture dish. Three-dimensional aggregate was formed only in the F-dish. The advantage of three-dimensional aggregate for albumin secretion was not clear compared with two-dimensional monolayer.

Introduction

Primary culture of hepatocyte is generally performed as a monolayer culture in a petri dish with coating type I collagen. Commonly used medium depth is 2–5 mm in the petri dish, and stationary dish culture is performed in a 5% CO_2 :20% O_2 atmosphere. Under such culture condition, medium layer over the cells becomes resistance of oxygen transport (McLismans et al., 1968). It has been reported that medium depth should be not greater than 0.34 mm to maintain hepatocyte culture in the dish (Stevens, 1965), but the depth of culture medium becomes over 1 mm for reasons such as prevention of drying and supply of nutrition. In primary hepatocyte cultures, oxygen supply is a critical issue because the highly oxygen demanding hepatocytes are maintained in the dishes under oxygen-deficient culture condition and are thus forced into anaerobic metabolic state. The stationary dish culture of hepatocytes can not express the sufficient liver function due to the oxygen deficiency (Catapano et al.,

1996). Despite there is such critical issue of oxygen supply, the stationary dish culture is still employed for *in vitro* culture experiment of primary hepatocytes. For example, in the study on the development of bioartificial liver, the stationary dish culture has been used as control cultures to evaluate the liver function expression (Miyoshi et al., 1994). Hepatocyte cultures under oxygen-deficient condition seem to be unsuitable as a control culture for the estimation of metabolic activity.

The purpose of this study is to clarify the degree of liver function depression in the stationary dish culture of primary hepatocytes. We performed primary cultures of rat hepatocyte in an oxygen-permeable film dish, which would be expected to give an oxygen-rich culture condition, as comparison with the conventional dish cultures, and discussed the importance of the oxygen for primary hepatocytes to express the liver-specific function.

Figure 1. Typical oxygen profiles in PC dish and F-dish just after seeding of hepatocyte. The cell density was 1×10^5 cells cm^{-2} in 0.2 ml medium. The depth of culture medium was 2 mm. Symbols: $\circ$, PC-dish; $\bullet$, F-dish.

Materials and methods

Isolation of rat hepatocytes

Hepatocytes were isolated from male Wistar rats (Japan SLC Inc., Shizuoka), weighing 180 to 200 g, by a two-step *in situ* collagenase perfusion technique (Seglen, 1976). The cell suspension was centrifuged at $50 \times g$ for 30 s. The cell pellet purified in the further centrifugation of 2 times was used as hepatocytes. Routinely, $2.5\text{--}3.0 \times 10^8$ hepatocytes were obtained from a single isolation with a viability of 85–95% as determined by the exclusion of Trypan blue dye.

Culture dishes

Culture dishes used were 60 mm polystyrene dish coated with collagen type I (PC-dish, Iwaki) for conventional stationary dish cultures and 60 mm dish with bottom made from oxygen-permeable film (F-dish, Hereaus) for oxygen enrichment cultures. It was possible to supply the oxygen from the dish bottom in the F-dish. The material of an oxygen-permeable

film was polytetrafluoroethylene (PTFE, Viva Science, Germany). The film thickness was $25 \mu\text{m}$. The diffusion rate of oxygen was $6.3 \mu\text{mol cm}^{-2} \text{hr}^{-1}$ at 0.18 atm for pressure gradient. Following dishes were otherwise used for the comparison experiment, which were 60 mm polystyrene dish without collagen (P-dish, Corning), the F-dish coated with collagen (FC-dish), and the F-dish covered its bottom with polystyrene plate (FP-dish). In the FP-dish, the polystyrene plate disturbed the oxygen supply from the dish bottom. In all dishes, effective surface area for culture was about 20 cm^2 .

Hepatocyte culture

The isolated hepatocytes were incubated in Williams' medium E (WE medium, Gibco BRL, Paisley, UK) supplemented with 10^{-7} M insulin, 10^{-7} M dexamethasone, 20 ng ml^{-1} EGF, 50 mM linoleic acid, $0.1 \text{ mM CuSO}_4 \cdot 5\text{H}_2\text{O}$, $3 \text{ nM H}_2\text{SeO}_3$, 50 pM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 15 mM HEPES, 100 units ml^{-1} penicillin, $100 \mu\text{g ml}^{-1}$ streptomycin, and 2.2 g l^{-1} NaHCO_3 . The hepatocytes were seeded in the culture

Figure 2. Albumin secretion of hepatocyte cultures in PC-dish and F-dish. Using F-dish had significant effect on the albumin production ($P < 0.05$). Each bar represents the mean $\pm$ SD of four wells. Symbols: $\circ$, PC-dish; $\bullet$, F-dish.

dishes to give 1×10^5 cells cm^{-2} in 0.2 ml medium. Medium volume poured into the dish was 4 ml. The depth of culture medium became 2 mm. The stationary cultures were conducted at 37°C in a 5% CO_2 :20% O_2 atmosphere under a humidified condition. They were cultured with daily medium change.

Measurement of oxygen tension in culture medium

Oxygen tension (pO_2) in the culture medium was measured with a fluorescent oxygen probe (FO-960, Automatic System Research Co., Ltd., Tokyo). The probe tip was set to the position of 1 mm from the bottom of dish, and 4 ml of cell suspension (5×10^5 cells ml^{-1}) was poured into the dish. The pO_2 change from just after seeding was monitored until the steady state was obtained under the culture condition mentioned above. After 2 days of culture, the pO_2 was measured by inserting the probe into the medium of culture state. The pO_2 is indicated as percentage of partial pressure of dissolved oxygen against saturated one under the pure oxygen gas atmosphere. The pO_2 was about 20% under ordinary atmosphere.

Biochemical assays

As indexes of the liver functions, the activities of albumin secretion, urea synthesis, and ammonium metabolic activity were measured. The amount of albumin in the medium was measured by enzyme-linked immunosorbent assay (ELISA) with purified rat albumin (Cappel, Durham, NC) and peroxidase-conjugated antibody (Cappel, Durham, NC) (Dunn et al., 1991). Urea nitrogen concentration in the medium was measured colorimetrically by diacetyl monoxime method (Coulombe and Favreau, 1963). Ammonia removal activity was determined by the initial velocity assay. The culture medium was replaced with the medium containing 1 mM NH_4Cl , and the initial rate of decreasing ammonia concentration was measured colorimetrically by indophenol method (McCullough, 1967).

Statistics

For the measurement of albumin, experiments were performed twice with four measurements per experiment. Statistical analyses were performed using the t-

Figure 3. Time course changes in the accumulation of albumin in both cultures of PC-dish and F-dish during the initial 72 hr without medium change. Using F-dish had significant effect on the albumin production ($P < 0.05$). Each bar represents the mean $\pm$ SD of four wells. Symbols: $\circ$, PC-dish; $\bullet$, F-dish.

Table 1. Change of oxygen tension in each culture dish during the culture period

Culture period	Oxygen tension: pO ₂			
	PC-dish		F-dish	
	(%)	(mmHg)	(%)	(mmHg)
Just after seeding	0.3	2.3	8.5	64.6
Two days postseeding	0.7	5.3	15.1	115

(2.3 mmHg) within 2 hr, while the pO₂ in the F-dish maintained 8.5% (64.6 mmHg) even after 2 hr. In 2 days postseeding, when hepatocytes attached and spread onto the surface of dish bottom, the pO₂ in steady state rose in both dishes: pO₂ in the PC-dish was 0.7% (5.3 mmHg) and pO₂ in the F-dish was 15.1% (115 mmHg). These measured pO₂ values are summarized in Table 1. It was shown that the F-dish could offer more aerobic culture condition than the PC-dish.

test at a significance level of 0.05. Error bars represent the standard deviation of the mean.

Results

Oxygen tension in culture dish

Typical pO₂ changes from just after seeding of hepatocytes are shown in Figure 1. The pO₂ in the PC-dish decreased from 19% (144 mmHg) to 0.3%

Albumin secretion

Time course changes in albumin secretion activity of the F-dish and the PC-dish are shown in Figure 2. The hepatocytes cultured in the F-dish showed higher albumin secretion activity than that in the PC-dish. The F-dish allowed the hepatocytes to express adequate albumin secretion activity from the culture day 1 and the activity was relatively stable during culture period. In

Figure 4. Ammonia elimination of hepatocyte cultures in PC-dish and F-dish. Symbols: $\circ$, PC-dish; $\bullet$, F-dish. Each value is the mean of two independent experiments.

Figure 5. Difference in cellular morphology on day 4 between PC-dish and F-dish. (a) Phase-contrast microscopic features of rat hepatocytes cultured in PC-dish (original magnification $\times 100$). (b) Phase-contrast microscopic features of rat hepatocytes cultured in F-dish (original magnification $\times 40$).

the culture day 1, four times albumin of the PC-dish was secreted by the hepatocytes in the F-dish.

Figure 3 shows the time course changes in the accumulation of albumin during the initial 48 hr without medium change. In the F-dish, albumin concentration linearly increased in the medium until 48 hr. The average rate of albumin secretion in this period was $2.0 \mu\text{g ml}^{-1} \text{hr}^{-1}$. In the PC-dish, the albumin secretion rate in the initial 12 hr was low ($0.36 \mu\text{g ml}^{-1} \text{hr}^{-1}$), and it gradually increased up to $1.5 \mu\text{g ml}^{-1} \text{hr}^{-1}$ in the culture period of 24–48 hr. It was found that the hepatocytes cultured in the F-dish demonstrated the sufficient activity of albumin secretion from just after seeding.

Ammonia elimination and urea synthesis

The rate of ammonia elimination for the initial 4 hr was measured every 24 hr. As shown in Figure 4, the activity of ammonia elimination in the F-dish was 20–50% higher than that in PC-dish during culture period, but the activity difference between both the dishes was not so remarkable as the albumin secretion. The significant difference could not be recognized in urea synthesis between both the dishes (data not shown).

Morphological appearance

Figure 5 shows the difference in cellular morphology on day 4 between the PC-dish and the F-dish. In the early period of the cultures, hepatocytes adhered on the dish bottom and expended, and then formed monolayer in both the dishes. The PC-dish maintained the monolayer form during culture period (Figure 5a). In the F-dish, the hepatocytes began to aggregate from day 3, and the cell aggregates liberated from the dish bottom on day 4 (Figure 5b). Liberated cell aggregates also exhibited the activities of albumin secretion, ammonia elimination, and urea synthesis.

Albumin secretion in various culture dishes

Various cultures shown in Table 2 were carried out in order to clarify that the oxygen is an essential to raise the albumin secretion. Whether collagen was coated on the film (FC-dish) or not (F-dish), the dish using a film was more advantageous than other polystyrene dishes (P-dish and PC-dish) for the albumin secretion. But, when the oxygen transfer through the film was disturbed (FP-dish), the activity of albumin secretion became equivalent level to that in the polystyrene dishes. The hepatocytes cultured in the

FC-dish did not aggregate and the formed monolayer was maintained during culture period. In the FP-dish, the monolayer form was observed, but the cell aggregation did not happen. It was found that favorable albumin secretion was observed under the oxygen-rich culture condition using the F-dish or the FC-dish.

Discussion

It has been stated that the oxygen tension ($p\text{O}_2$) of pooled venous blood is about 40 mmHg while that of arterial blood is about 95 mmHg (Stevens, 1965). Therefore the $p\text{O}_2$ to which liver cells are exposed *in vivo* should be between these values. As shown in Table 1, the $p\text{O}_2$ in the conventional culture dish (PC-dish) was considerably lower than 40 mmHg. Schumacker et al (1993) have reported that about 10 mmHg is the critical $p\text{O}_2$ for the oxygen uptake rate (OUR) of hepatocytes. The OUR of hepatocytes is high during the early period of culture and decreases after the initial cellular reorganization period (Rotem et al., 1992). In this study, the $p\text{O}_2$ was 5.3 mmHg in the PC-dish even after the initial cellular reorganization period. Below the critical $p\text{O}_2$, the metabolism of hepatocytes is limited due to oxygen-deficiency. As shown in Figures 2–4, the hepatocytes cultured in the oxygen-permeable film dish (F-dish) showed higher activities of albumin secretion and ammonia elimination than those cultured in the PC-dish. Especially, the remarkable difference of albumin secretion was observed in the early period of culture between the F-dish and the PC-dish (Figures 2 and 3). The average rate of albumin secretion for the initial 48 hr was $2.0 \mu\text{g ml}^{-1} \text{hr}^{-1}$ or $96 \mu\text{g } 10^6 \text{ cells}^{-1} \text{day}^{-1}$ in the F-dish. This value was almost equivalent to albumin secretion rate of rat organism liver (Peters Jr and Peters, 1972). In the PC-dish, the average rate of albumin secretion for the initial 12 hr was only $0.36 \mu\text{g ml}^{-1} \text{hr}^{-1}$. This seems to be because the F-dish could maintain the oxygen level above the critical $p\text{O}_2$ for OUR in the early culture period.

In the early period of culture, the hepatocytes adhered and spread onto the oxygen-permeable film with or without collagen coating, and then the spreading cells formed the monolayer of two-dimensional cellular reorganization. The reorganized form of hepatocytes is regarded as an appropriate physical state for albumin secretion. The morphology of the monolayer observed in the F-dish resembled that in the PC-dish, but the activity of albumin secretion in the F-dish was

Table 2. Comparison of albumin activity in various culture dishes

Dish type	Matrix	Albumin secretion ($\mu\text{g } 10^{-6} \text{ cells} \cdot \text{day}$)	
		Average value ^a ($\pm$ SD)	Peak value ($\pm$ SD)
Oxygen permeable film dish (F-dish)	no	77.4 ($\pm$ 3.1)	106.2 ($\pm$ 5.1)
Oxygen permeable film dish (FC-dish)	collagen	101.2 ($\pm$ 5.2)	107.6 ($\pm$ 3.2)
Covered the bottom of F-dish with polystyrene plate (FP-dish)	no	59.7 ($\pm$ 2.7)	67.4 ($\pm$ 0.6)
Polystyrene dish (P-dish)	no	55.2 ($\pm$ 3.0)	72.6 ($\pm$ 2.2)
Polystyrene dish (PC-dish)	collagen	43.1 ($\pm$ 0.8)	66.2 ($\pm$ 0.4)

^a Average value of 7 days culture.

high regardless of the similarity of the cell morphology. It has been reported that the oxygen tensions of 0.064 and 0.13 mmHg are required for the half-maximal attachment of hepatocytes to collagen-based substrate and for the spreading process, respectively (Rotem et al., 1994). These values are rather lower than the critical value for OUR. Since the hepatocytes required more oxygen for albumin secretion than for the formation of monolayer, little albumin was secreted in the early culture period of the PC-dish even if two-dimensional cellular reorganization was formed (Figure 3).

In the late culture period of the F-dish, the monolayer was torn from the dish bottom and three-dimensional aggregates were formed. It is considered that the three-dimensional cell aggregate is the form that is more advantageous than the two-dimensional monolayer one for the liver function expression (Koide et al., 1989, 1990; Tobe et al., 1992). Koide et al. (1989) has reported that spherical cell aggregates (spheroids) maintain relatively high albumin production activity for longer period to the monolayer one under the conventional culture condition. As shown in Table 2, however, there was no clear difference in albumin secretion between the two-dimensional monolayer (FC-dish) and the three-dimensional aggregates (F-dish). This discrepancy seems to be due to the degree of oxygen satisfaction in the culture.

The cells in the spheroid must be well differentiated than those in the monolayer. Therefore, it is supposed that some of liver functions demonstrated in the monolayer are inferior to the spheroid. However, the cells in the monolayer are thought to be well-differentiated for albumin secretion. As described here, the monolayer secreted comparable amount of albumin to the spheroid when the cultivation was performed with sufficient oxygen supply. It is estimated that the oxygen uptake rate of the cells in monolayer

is higher than that in spheroid, because the oxygen uptake rate tends to be lower with the advance of the cell differentiation (Wu et al., 1996). Therefore, the monolayer is frequently cultivated under the condition of oxygen-deficiency further than the spheroid in the conventional culture using petri dish. Since the oxygen is an important factor to affect the albumin secretion, the difference in the degree of oxygen satisfaction between the monolayer and the spheroid might appear in the albumin productivity in the previous reports.

From the above results, it is clear that the oxygen-deficiency in the conventional culture dish suppressed albumin secretion. The problem of the oxygen-deficiency was more remarkable in the early period of culture. Therefore, other liver functions may be also suppressed in the conventional culture dish. Effect of oxygen on the metabolism should be more considered in the primary hepatocyte culture using petri dish.

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