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An *in vitro* hepatic zonation model with a continuous oxygen gradient in a microdevice

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ABSTRACT

In a hepatic lobule, different sets of metabolic enzymes are expressed in the periportal (PP) and pericentral (PC) regions, forming a functional zonation, and the oxygen gradient is considered a determinant of zone formation. It is desirable to reproduce lobular microenvironment *in vitro*, but incubation of primary hepatocytes in conventional culture dishes has been limited at fixed oxygen concentrations due to technical difficulties.

We designed a cell culture microdevice with an oxygen gradient to reproduce the hepatic microenvironment *in vitro*. The oxygen gradient during cell culture was monitored using a laser-assisted phosphorescence quenching method, and the cellular oxygen consumption rate could be estimated from changes in the gradient. Culture medium was continuously exchanged through microchannels installed in the device to maintain the oxygen gradient for a long term without transient hyper-oxygenation.

The oxygen consumption rates of hepatocytes at 70.0 mmHg and 31.4 mmHg of partial oxygen pressure, which correspond to PP and PC regions in the microdevice, were 3.67×10^{-10} and 3.15×10^{-10} mol/s/10^6 cells, respectively. Antimycin A changed the oxygen gradient profile, indicating that cellular respiration can be estimated during cell culture. RT-PCR analysis of hepatocytes cultured under the oxygen gradient showed that mRNA expression of PEPCK and GK significantly increased in culture areas corresponding to PP and PC regions, respectively.

These results indicate that the developed microdevice can reproduce the hepatic lobular microenvironment. The oxygen gradient in the microdevice can be closely controlled by changing the sizes of gas channels and the ambient oxygen concentration around the device; therefore, it could be expected to mimic the oxygen gradient of various organs, and it may be applicable to other pathological models.

1. Introduction

In a hepatic lobule, parenchymal cells do not necessarily perform the various metabolic functions homogeneously, but they play different roles according to zonation in the lobule [1,2]. Enzymes involved in gluconeogenesis and urea synthesis are predominantly expressed in the periportal (PP) region, whereas enzymes involved in glycolysis, detoxication, and drug metabolism are expressed in the pericentral (PC) region of the liver [3]. Zone specificity of metabolism has not been recognized in the fetus, but it is considered to be an important factor determining the formation by hormones, nutrients, oxygen, and other substances in portal blood flow of a concentration gradient between the PP and PC regions of the hepatic lobules in response to dietary intake at the incipient stage [4]. The oxygen gradient in the lobule is generated by oxygen diffusion and exchange in sinusoids. It is effectively impossible to reproduce the hepatic lobule, in which metabolic functions differ partially *in vitro*, using a general-purpose cell culture dish and an incubator. An oxygen gradient can be generated in a direction of flow by perfusing the medium of cultured hepatocytes that consume oxygen [5]. However, since the oxygen solubilities of blood and culture medium differ substantially, it is difficult to control the oxygen gradient to reproduce physiological and pathological conditions.

Abbreviations: PP, periportal; PC, pericentral; MEMS, microelectromechanical systems; pO_2, partial oxygen pressure; GK, glucokinase; PEPCK, phosphoenolpyruvate carboxykinase; OCR, oxygen consumption rate.

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Oxygen concentration in blood supplying the liver is lower than that in other organs because approximately 70% of the hepatic blood supply is poorly oxygenated blood derived from the portal vein [6]. In addition, hepatocytes consume relatively large amount of oxygen [7]; therefore, the liver has a considerable risk of hypoxia [8,9]. For instance, it is known that increased oxygen consumption in alcohol metabolism induces tissue hypoxia downstream in the hepatic lobule, resulting in partial tissue injury in the PC region [10]. In general, to reproduce tissue hypoxia *in vitro*, the oxygen concentration in an incubator is reduced; however, long term culture under hypoxic condition is technically difficult because experimental results differ with 1–2% differences in oxygen concentration set points, and repeated transient oxygen exposures occurring with medium replacement are unavoidable. It has been reported that differences of urea synthesis in hepatocyte populations according to oxygen concentration took at least 5 days [11]; therefore, long term culture is essential for cellular remodeling to form metabolic zonation *in vitro*.

In the present study, we developed a novel cell culture microdevice using MEMS. The device can generate controllable oxygen gradients in culture layers, expose cells simultaneously to a wide range of oxygen concentrations from hyperoxia to hypoxia or to physiological conditions, and exchange the culture medium automatically to allow long-term culture, with a stable oxygen gradient. The cellular oxygen consumption rate (OCR) can be estimated during cell culture by measurement of partial oxygen pressure (pO_2) using a laser-assisted phosphorescence quenching method. If molecular remodeling in hepatocytes can be induced by long-term culture under an oxygen gradient, metabolic zonation close to a hepatic lobule can be mimicked *in vitro*. This capability could be applied in pathological models, pharmacological evaluation, and studies of artificial livers.

We performed pO_2 measurement to quantify the oxygen gradient along with oxygen diffusion simulation to evaluate the feasibility of the microdevice and estimated the OCR of primary cultured hepatocytes. We also evaluated the mRNA expression levels of PEPCK and GK to confirm that the microdevice can mimic the *in vitro* microenvironment of hepatic zonation.

2. Materials and methods

2.1. Design and fabrication of a cell culture microdevice forming an oxygen gradient

The cell culture microfluidic device that generates an oxygen gradient has a bilayer structure, with a cell culture layer forming an oxygen gradient and an automated medium exchange layer (Fig. 1A). Each unit is made of polydimethylsiloxane (PDMS), with high gas permeability and biocompatibility [12], and fabricated photolithographically. The oxygen gradient in the cell culture layer is formed by oxygen diffusion from an air–gas (20% O_2 and 5% CO_2) channel with cross-sectional dimensions of 400 × 500 μm in the cell culture layer (Fig. 1B). Pd-meso-tetra (4-carboxyphenyl) porphyrin (Pd-TCPP) is mixed with PDMS. The mixture is manufactured to a thickness of 80 μm, bonded on the top of the substrate, and used as an oxygen sensor [13]. Cells are cultured on the oxygen sensor film, allowing the monitoring pO_2 and estimation of cellular OCR during culture. An upper layer is mounted on the cell culture layer and filled with culture medium. To preserve the stable oxygen gradient for a long period of time without transient hyper-oxygenation by medium exchange, fresh culture medium flows into the device continuously from two microchannels at a flow rate of 13.8 μL/min, and an equivalent amount of old culture media is discharged. All PDMS parts and a glass substrate are assembled by oxygen plasma bonding. The cell culture device is set in a gas-tight chamber (Fig. 1C and D), and the oxygen pressure in the chamber is maintained at 30 mmHg.

2.2. Hepatocyte isolation and primary culture in the microdevice

All animal experimental protocols were approved by the Animal Care Committee of Keio University School of Medicine. Hepatocytes were isolated from male BALB/c mice weighing 25–30 g by digestion of livers with type I collagenase as described previously [14]. All perfusions were performed at flow rates of 7 mL/min and all buffers were maintained at 37 °C. The first buffer consisted of 20 mL of Hank's–HEPES solution containing 1 mM EGTA, and the

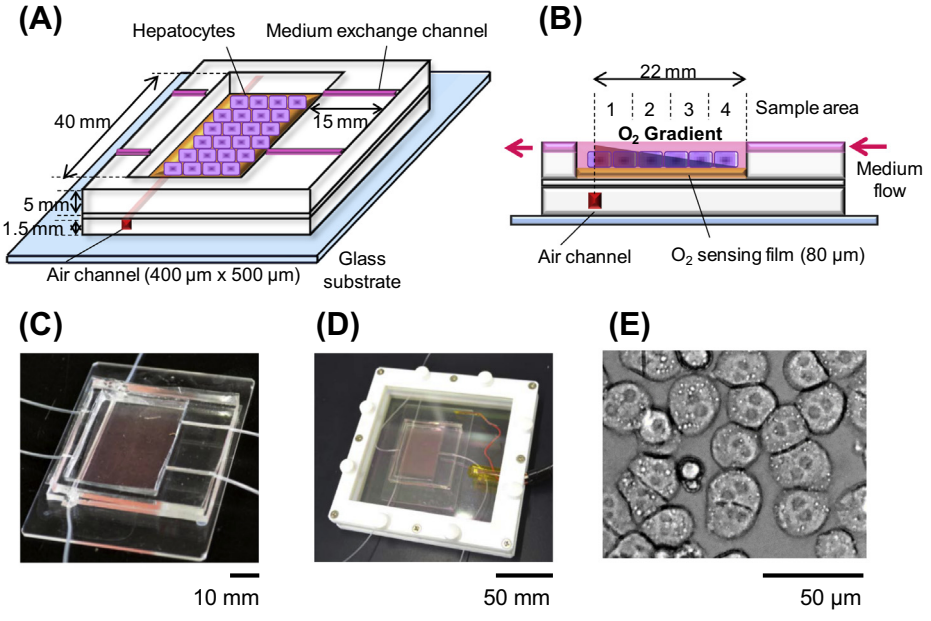

Fig. 1. Schematic view (A) and cross-sectional view (B) of a cell culture microdevice that generates an oxygen gradient. The culture medium in a microdevice is automatically exchanged via two sets of flow tubes for long-term culture (C). The device is set in a gas-tight chamber to maintain low ambient oxygen (D). Oxygen tension around primary cultured hepatocytes and their oxygen consumption can be monitored with an oxygen sensing film (E).

second buffer consisted of 20 mL of Hank's–HEPES containing collagenase I (0.5 mg/mL, Wako Pure Chemical Industries, Japan). Hepatocytes were purified by washing twice with ice-cold Hank's–HEPES buffer solution followed by Percoll gradient separation. Primary hepatocytes were seeded on the collagen-coated PDMS film at a density of 6.4×10^4 cells/cm^2 in William's medium E containing 10% fetal bovine serum, 100 nM insulin, 100 nM dexamethasone, and epidermal growth factor (EGF, 20 ng/mL). The insulin content was reduced to 1 nM for analysis of PEPCK, and the insulin and FBS were reduced to 0.5 nM and 4%, respectively, for analysis of GK [15,16]. For the oxygen gradient measurement, 50 μM of antimycin A was added to the medium to reduce cellular respiration. For PCR analysis, the cell culture area from the air channel to its edge was divided into four regions as shown in Fig. 1B, and hepatocytes were sampled after incubation for 28 h under an oxygen gradient.

2.3. Oxygen gradient measurement and estimate of oxygen consumption rate of hepatocytes

Oxygen tension around cultured cells was measured using the oxygen sensor film as described previously [13]. The Pd-TCPP in the oxygen sensing film was irradiated with exciting light from a Q-switched Nd:YAG pulse laser (wavelength, 532 nm; pulse width at half maximum, 5 ns; pulse recurrence frequency, 10 Hz; irradiation energy, 2.5 μJ/pulse) through an objective lens, and emitted phosphorescence was detected with a photomultiplier mounted on the microscope. The lifetime of the phosphorescence, which depends on the oxygen concentration, was measured from just above the air channel to the end of the culture area at intervals of 1 mm, and the pO_2 was obtained from the lifetime by the Stern–Volmer Eq. (1):

$$\frac{\tau_0}{\tau} = 1 + k_q \tau_0 [pO_2] \tag{1}$$

The diffusion Eq. (2) and the difference method shown in Eq. (3) were applied to estimate the oxygen gradient mathematically in the device:

$$\frac{\partial P(t,x,y)}{\partial t} = D\left(\frac{\partial^2 P(t,x,y)}{\partial x^2} + \frac{\partial^2 P(t,x,y)}{\partial y^2}\right) \tag{2}$$

$$\begin{aligned} P(t+\Delta t,x,y) = {} & P(t,x,y) \\ & + \frac{D\Delta t}{\Delta x^2}\{P(t,x-\Delta x) - 2P(t,x) + P(t,x+\Delta x)\} \\ & + \frac{D\Delta t}{\Delta y^2}\{P(t,y-\Delta y) - 2P(t,y) + P(t,y+\Delta y)\} \\ & - NC \end{aligned} \tag{3}$$

where P and D are partial oxygen pressure and diffusion coefficient, respectively. We set the diffusion coefficient of oxygen in water at 2.1×10^{-3} mm^2/s and that in PDMS at 4.1×10^{-3} mm^2/s [17], respectively. The time step Δt was 0.15 s and the length step Δx, y was 50 μm, the initial condition was 159 mmHg, and the boundary condition was 30 mmHg using Dirichlet conditions. An oxygen consumption term NC was added only to the computational cell corresponding to the culture layer in the device; the cell density N was set to 6.4×10^4 cells/cm^2 and the oxygen consumption C to 2.50×10^{-10} mol/s/10^6 cells [18]. The relationship of the OCR to the magnitude of shift of the gradient was preliminarily calculated by simulation, and the OCR of cultured cells was estimated by applying the actually measured pO_2 shift to the simulation results. All programs were written in the C^{++} language.

2.4. qRT-PCR

Total RNA from primary cultured hepatocytes was extracted using an RNeasy kit (Qiagen, USA). Quantitative real-time RT-PCR was performed using the Light-Cycler Nano System (Roche, Germany) with a standard temperature protocol and PrimeScript RT reagent kit (Takara Bio, Japan). As an internal control, β-actin was used for normalizing, and data were presented as relative mRNA variation. The primer sequences were as follows: mouse PEPCK forward 5′-AGCCTTTGGTCAACAACTGG-3′, reverse 5′-TGCCTTCG GGGTTAGTTATG-3′; mouse GK forward 5′-TATGAAGACCGCCAAT GTGA-3′, reverse 5′-CACTGAGCTCTCATCCACCA-3′; and mouse β-actin, forward 5′-AGAAAATCTGGCACCACAC-3′ reverse 5′-TTTGA GACCTTCAACAC-3′.

2.5. Statistical analysis

All data are presented as mean ± S.E. Mean values were compared by Student's t test where appropriate. $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Oxygen gradient in the microdevice and oxygen consumption rate of cultured cells

Primary cultured hepatocytes in the microdevice showed morphologies similar to those in conventional culture dishes, as shown in Fig. 1E. The oxygen sensor film on the cell culture layer allowed monitoring pO_2 during culture. When the oxygen gradient reached steady state, at 24 h after cell seeding, it was measured as shown in Fig. 2A. The profile of the oxygen gradient was shifted down compared with the non-cell culture, indicating that cells consumed oxygen by respiration. Moreover, addition of antimycin A, an inhibitor of the mitochondrial respiratory chain, reduced the size of the downward shift. In cells with higher mitochondrial density, particularly in hepatocytes, the downward shift of the gradient with oxygen consumption is large compared with that in other cells, implying that the oxygen consumption of cells can be estimated from the oxygen gradient shift. The OCR of hepatocytes at pO_2 70.0 mmHg and 31.4 mmHg, which correspond to the PP and PC regions in a hepatic lobule, were 3.67 and 3.15 mol/s/10^6 cells, respectively, as shown in Table 1. The shift amount of pO_2 was larger in area 3, corresponding to the PC region, but oxygen consumption simulated by the oxygen diffusion was slightly larger in area 2, the PP region. This difference in the OCR may reflect a difference in the respiration activity corresponding to the oxygen concentration around cells rather than a difference in mitochondrial density, given that 24 h of culture is too short for mitochondrial remodeling. Antimycin A equally decreased pO_2 in the PP and PC regions, but simulated OCR was much lower in the PC than in the PP region, indicating that the cellular respiration should be estimated by the oxygen diffusion and not only by the pO_2 difference.

We previously reported the pO_2 measurement in portal venules, sinusoids, and central venules *in vivo*, and the oxygen gradient showed a linear decrease in hepatic lobules [19]. In our microdevice, pO_2 decreased linearly from areas 2 to 3, corresponding to the PP and PC regions, indicating that the device mimics the hepatic microenvironment. However, there were differences between the actual measurement (plots in Fig. 2A) and simulated gradients (dashed lines in Fig. 2A). Adjusting the diffusion constants of the materials of the device and accounting in the diffusion simulation for medium flow in the culture area could improve simulation.

Cultured cells in the device were divided into four areas. In addition to areas 2 and 3 near physiological conditions, cells can

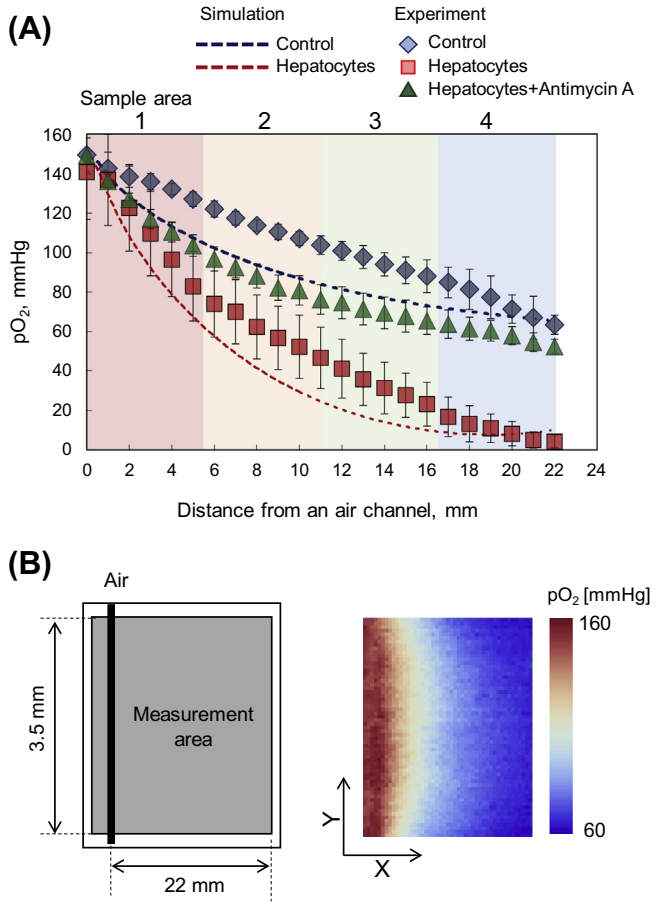

Fig. 2. The oxygen gradient in the culture layer was measured (A). Primary cultured hepatocytes shifted the gradient by their respiration (square dot) compared to control (without cell, rhombic dot). Antimycin A inhibited the respiration and decreased the amount of the shift (triangular dot). The gradient was predicted by simulation using a diffusion equation (dotted lines). The sample area was divided into four areas, with areas 2 and 3 corresponding to the PP and PC regions, respectively. The oxygen gradient in the culture layer is visualized in pseudocolor by scanning pO_2 in it (B).

Table 1
Cellular oxygen consumption rates at positions corresponding to the PP and PC regions.

Distance from the air channel (mm)	Antimycin A	pO_2 (mmHg)	pO_2 shift (mmHg)	OCR (mol/s/10^6 cells)
7 (equal to PP region)	–	70.0	47.5	3.67×10^{-10}
	+	92.6	24.9	2.07×10^{-10}
14 (equal to PC region)	–	31.4	62.7	3.15×10^{-10}
	+	69.5	24.6	0.77×10^{-10}

be simultaneously subjected to hyper-oxygenation (area 1) and hypoxic conditions (area 4) for disease modeling. The results of the pO_2 scanning of the cell culture layer (Fig. 2B) revealed a uniform pO_2 gradient in the *y*-axis direction. In future, establishing an oxygen gradient along the *x*-axis and a hormone or drug gradient along the *y*-axis may reduce time and cost of experiments by allowing the evaluation of multiple conditions at once. The microdevice can mimic oxygen gradients in various organs if numbers, sizes, and locations of gas channels and O_2 concentration in the gas-tight chamber are changed [13,20]. For instance, placing one N_2 channel between two O_2 channels in parallel can form a V-shaped oxygen gradient, which can be used in a bone marrow model. The profile of the oxygen gradient may be approximately predicted by simulation with the diffusion equation, and the laser-assisted pO_2 measurement allows rapid and accurate establishment of various O_2 gradients in the cell culture microdevice.

3.2. Long-term cell culture under an oxygen gradient

Our cell culture microdevice can maintain the oxygen gradient over a long term by automatic medium exchange. Fresh culture medium constantly flows in through two channels of 15 mm length from inlets on the microdevice at a flow rate of 13.8 μL/min. Oxygen in the fresh medium diffuses while passing through the channel, and pO_2 of the medium at the end of the channel decreases to that of the gas-tight chamber. These flow conditions maintain a steady oxygen gradient for long-term cell culture even with infusion of medium. We visualized the diffusion and convection of trypan blue-supplemented new medium flowing into the old medium in the device and found that approximately 3 h were required for uniform distribution in the culture area (data not shown). In conventional cell incubation in hypoxic research, transient oxygen exposure with medium replacement is unavoidable, and it is well known that oxygen exposure following hypoxia induces oxygen stress by generation of reactive oxygen species. The device can maintain prolonged hypoxic conditions without impulse stimulation by oxygen. Hepatocytes are particularly suitable for long-term culture because of their comparatively longer cell division period. Long-term culture under an oxygen gradient offers possibility of changing the molecular expression in hepatocytes with the oxygen gradient, affording even better mimicking of *in vivo* hepatic lobules. HIF-1 has been reported to regulate the mitochondrial biogenesis [21]. However, no technique has been proposed to measure the mitochondrial density and oxygen consumption simultaneously under various oxygen concentrations. Our cell culture microdevice may be expected to be used to elucidate previously unrevealed phenomena, such as the relationship between liver regeneration and oxygen consumption.

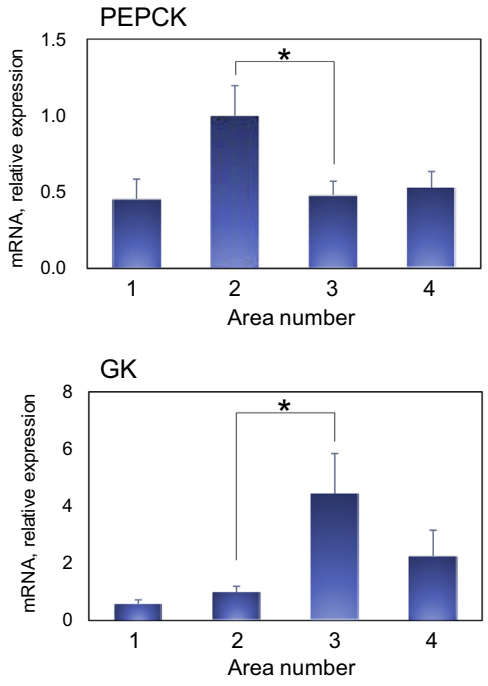

Fig. 3. Quantitative analysis of mRNA expression of PEPCK and GK mRNA as typical metabolic enzymes, expressed predominantly in the PP and PC regions, showed a significant increase of PEPCK in area 2 and of GK in area 3 of the microdevice, corresponding to the PP and PC regions. $^*p < 0.05$.

3.3. Zone-specific expression of metabolic genes

It is impossible to reproduce the variation of expression of metabolic enzymes among the PP and PC regions using a general-purpose culture dish. PEPCK and GK *in vivo* are expressed predominantly in the PP and PC regions, respectively and regulated by oxygen tension. RT-PCR analysis of mRNA expression of PEPCK and GK in hepatocytes cultured under an oxygen gradient showed significant increase of PEPCK in area 2 (corresponding to the PP region) and GK in area 3 (corresponding to the PC region) as shown in Fig. 3. It is also possible to sample cells simultaneously from areas 1 and 4, which correspond to hyper-oxygenation and hypoxic conditions, as well as from areas 2 and 3, which correspond to near-physiological conditions. As the intracellular ROS generation will be increased in the hyper-oxygenated area, association between ROS and cell damage can be assessed. A subject for future investigation is whether ROS reduces the expression of PEPCK, despite the higher oxygen in area 1. In contrast, the hypoxic area can mimic pathological conditions, such as ischemia reperfusion or chronic hypoxia, simultaneously with physiological conditions. We hope to apply the cell culture microdevice to zone-specific hepatotoxicity research that is difficult to reproduce *in vitro*; for example, alcoholic liver disease. Adding other gradients, such as of hormones, to that of oxygen could be of future help in establishing artificial livers.

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