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Oxygen-permeable membrane-based direct oxygenation remarkably enhances functions and gene expressions of rat hepatocytes in both 3D and sandwich cultures

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ABSTRACT

Although there have been remarkable progresses in hepatocytes cultures in terms of mimicking microenvironments of *in vivo* liver, oxygen supply is still a critical issue. In this study, we investigated the effect of direct oxygenation through oxygen-permeable membranes on functionalities of hepatocytes in two widely accepted advanced culture models, sandwich culture and 3D culture. Rat hepatocytes were cultured on the polydimethylsiloxane (PDMS) membranes for 14 days in monolayer culture, sandwich culture with Matrigel and 3D culture with microporous expanded polytetrafluoroethylene (ePTFE) membranes in the presence and absence of direct oxygenation from the other side of the membranes. The present results showed remarkable enhancement of hepatocytes duration and their functions by oxygen transfer through PDMS membranes in all these three cultures. The hepatocytes cultured in sandwich with oxygen exhibited extended survival and highest maintenance of metabolic activities, such as albumin productivity and Cyp1a1/2 activity. Additionally, the expression levels of various drug-metabolism genes, as examined by PCR arrays, were also closest to those of freshly isolated hepatocytes. As the cellular maintenance has been greatly improved by microporous ePTFE membranes, the hepatocytes in 3D culture performed increased functions that comparable to those in sandwich culture. This study clearly illustrates that oxygenation is a critical factor to be considered in optimization of the microenvironments of hepatocytes cultures.

1. Introduction

The liver acts as the center of metabolism and is indeed one of the most important sites of drug ADME (absorption, distribution, metabolism and excretion)—efficiency/toxicity. With the majority of liver functions, including xenobiotic biotransformation, performed by hepatocytes, hepatocyte-based *in vitro* models are currently being used in the drug development. Consequently, the development and characterization of long-term cultures of hepatocytes as *in vitro* experimental models represent an important goal. A major problem, however, related to the hepatocytes and their *in vitro* cultures is the progressive loss of the hepatocellular phenotype in conventional monolayer cultures, at both morphological and functional levels [1,2]. To overcome these effects, two widely accepted cellular culture models have been developed, sandwich culture model and 3D culture model (such as spheroid culture model), to restore the metabolic functions over extended periods of cultivation [3], with advanced substrate [4] and further stimulation by adding suitable exogenous factors [5].

Sandwich culture has been a valid cellular model for the hepatocytes relying on the restoration of cell-extracellular matrix (ECM) contacts, which is a one of the key roles in the culture configurations of primary hepatocytes [4,6]. Using an overlay of Matrigel, a basement membrane extract from Engelbreth–Holm–Swarm (EHS) mouse sarcoma, could enable cells to express high functions while simplifying the culture model compared to the standard sandwich with two gel layers of type I collagen [7,8]. More recently, another advanced format, in which hepatocytes could be cultured in a 3D configuration to represent the cell-to-cell contact, has been developed. In 3D culture (such as spheroid culture model), the hepatocytes maintain not only *in vivo*-like morphological characteristics, but also liver-specific functionalities [9,10]. However, decreased functionalities were previously observed compared to those in sandwich culture, due to deficient cellular maintenance

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[11]. Expanded polytetrafluoroethylene (ePTFE), a microporous synthetic polymer, is one of the preferable substrates used to improve stability in culture according to firmly attached hemispheroids [12–14]. Based on ePTFE membranes, the quantitative comparison of hepatocyte functions between 3D culture and sandwich culture is highly valuable.

Oxygen is one of the critical exogenous factors for the hepatocytes *in vitro*. The oxygen consumption rate (OCR) of rat hepatocytes is quite high, almost 10-fold higher than that of other cell types, and 3-fold more during the attachment, spreading and reorganizing process right after the inoculation [15]. However, conventional tissue culture plates, such as the tissue culture treated polystyrene (TCPS), are incapable of sufficient oxygen supply as a result of the limited solubility of oxygen in the culture media, which force the hepatocytes to undergo hypoxia [16,17]. In our previous work, to solve this problem, we have developed a simple method of culturing cells on an oxygen-permeable membrane, polydimethylsiloxane (PDMS), to meet this intensive requirement for oxygen. Although there are several hepatocyte cultures using PDMS-based devices, such as microfluidic, PDMS bioreactors are integrated on the glass substrate. Thus, the diffusion through culture medium or not well-controlled perfusion is still likely to cause oxygen shortage at the cell layer. Our study of culturing hepatocytes direct on a PDMS substrate has barely been made [18]. The PDMS membranes could facilitate direct oxygenation by diffusion through the membranes, which is expected to meet the cellular OCR requirements and increase functional capabilities. As governed by Henry's law, the actual oxygen concentration at the cell level could be very close to the ambient concentrations [19,20].

In this study, we investigated the effect of direct oxygenation using PDMS membranes on functionalities of hepatocytes in sandwich culture with Matrigel and 3D culture with ePTFE membranes, where the cellular oxygen requirements could be completely meet. Various functional characteristics of the hepatocytes in each culture were systematically evaluated and compared. We aimed to explore a more reliable hepatocytes culture model with the sufficient oxygen supply to enhance hepatic metabolism and gene expression in a long duration for *in vitro* drug-screening research.

2. Materials and methods

2.1. Rat hepatocyte isolation and high-/low-oxygen cultures

Primary hepatocytes were isolated from 7- to 8-week old male Wistar rats (Sankyo Laboratory, Japan) weighing 200–300 g using a two-step collagenase perfusion technique, as described previously [21]. Only those cells with viabilities exceeding 85% after isolation were used. All animals were treated in compliance with the University of Tokyo guidelines for animal experiments, following the guidelines of the Japanese Ministry of Education. Hepatocytes were suspended in ice-cold seeding medium (Williams E with 10% FBS) and seeded at a density of 1.0×10^5 cells/cm^2. The hepatocytes were maintained at 37 °C in a humidified incubator in a 95% air and 5%CO_2 atmosphere. On day 1, the seeding medium containing unattached cells was aspirated, and culture medium containing Williams' Medium E (Gibco, Japan), 1% NEAA (Gibco, Japan), non-essential amino acids (Gibco, Japan), 0.1 μM insulin (Takara, Japan), 1 μM dexamethasone (Wako, Japan), 10 ng/ml mouse epidermal growth factor (EGF; Takara, Japan), 0.5 mM ascorbic acid 2-phosphate (from magnesium salt *n*-hydrate; Wako, Japan), 0.1 μM $CuSO_4$, 0.01 μM H_2SeO_3 and 1 μM $ZnSO_4$ was added. The Williams E-based culture medium was changed every two days.

To enable direct oxygenation by diffusion through PDMS membranes (high-oxygen cultures, PDMS-O_2 (+)), hepatocytes were cultured on a 0.5-mm-thick PDMS membrane assembled on a polycarbonate 24-well plate as Monolayer-O_2 (+) culture (Vessel, Japan) (Fig. 1A). PDMS discs were cut up and inserted into the wells of TCPS plates (Iwaki, Japan) as Monolayer-O_2 (−) culture to control the surfaces consistent as the low-oxygen cultures (PDMS-O_2 (−)) (Fig. 1B). Prior to cell culture, the PDMS surfaces were treated with oxygen plasma for 10 s, using a Reactive Ion Etching (RIE) machine (RIE-10NR; Samco, Japan), and coupled with aminosilane (Shinetsu Silicone, Japan). The introduced amino groups were reacted with the cross linker *N*-(4-maleimidobutyryloxy) succinimide (GMBS; Dojindo, Japan) and coated with ocean collagen. Conventional monolayer culture on collagen coated TCPS plates was employed as a control (TCPS-O_2 (−)) (Iwaki, Japan) (Fig. 1B).

2.2. Sandwich cultures with Matrigel and 3D cultures with ePTFE membranes

The seeding medium containing unattached cells in both Monolayer-O_2 (+) culture and Monolayer-O_2 (−) culture was aspirated on day 1, and culture medium supplemented with 150 μg/ml Matrigel was added as sandwich cultures (Sandwich-O_2 (+), Sandwich-O_2 (−)) (Fig. 1B) [15]. The Matrigel-supplemented culture medium was also changed every two days.ePTFE is a porous form of a synthetic fluoropolymer with a microstructure characterized by nodes interconnected by fibrils. The fibrils are length of 10–50 μm, interval of 2–5 μm, thickness of 50–70 μm and porosity of 90% (Vessel, Japan), which could restrict hepatocytes spread. The hepatocytes could initially attach on the ePTFE membranes and elongate to form cell layers by cell-to-cell connection. The elongated cells then started to retract and to form multicellular aggregates. The ePTFE membranes were washed with ethanol, and immersed in a coating solution of 1% poly-amino-acid urethane copolymer (PAU) and 0.1% ocean collagen in dichloroacetic acid, washed with water to remove the excess coating solution, dried, and sterilized with ethylene oxide [22]. Then ePTFE membranes were fixed on the PDMS membranes as a support, assembled on a polycarbonate 24-well plate (3D-O_2 (+)) (Vessel, Japan) (Fig. 1B). PDMS + ePTFE discs were cut up and inserted into the wells of TCPS plates (Iwaki, Japan) to keep the surfaces consistent as the low-oxygen cultures (3D-O_2 (−)). Fig. 1C shows the scanning electron microscopy (SEM; SM-300; F1 Topcon, Japan) picture of ePTFE.

2.3. Vertical cross-section analysis

On day 1 and day 14, cell-loaded PDMS + ePTFE substrates in 3D cultures were rinsed with PBS. After 1 h of fixation with 4% paraformaldehyde (PFA; Nacalai Tesque, Japan), the cell sheets on the PDMS + ePTFE membranes were rinsed with PBS (5 min, 3 times). Histological samples were prepared by the Saipaso Research Center (Tokyo, Japan) as follows: fixed samples were dehydrated, embedded in paraffin, then 5 μm thin vertical sections were cut off, deparaffinized, rehydrated, and stained with Hematoxylin and Eosin (HE). Images were taken with a transmitted light microscope (BX50; Olympus, Japan).

2.4. DNA quantification

Extracted DNA was quantified from the inoculated cells suspension and adherent cells on day 1 and day 14, which were scraped off and recovered in 1 ml PBS after rinsing twice with PBS to remove the unattached cells. The samples were sonicated for 20 s at 40 W in an ice bath. The DNA concentrations were measured using 4′,6-diamidino-2-phenylindole (DAPI; Dojindo, Japan) fluorometry [23]. Data represent the means ± SD ($n = 6$) from two independent experiments.