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Short Communication

Fluorous solvent effect on cells cultured on collagen membrane at the interface

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ABSTRACT

The culture of various kinds of anchorage-dependent cells (mouse melanoma B16, Mardin Darby canine kidney (MDCK), mouse embryonic fibroblast 3T3, and human hepatocarcinoma HepG2) on collagen membrane at the interface between the aqueous culture medium layer and fluorous solvent layer was investigated. Results showed that collagen membrane supported cell adhesion and proliferation during liquid–liquid interface culture. An increase in cell viability was observed, when cells were grown on a collagen membrane as compared with cells cultured in the absence of a solid scaffold. This research also confirmed that the fluorous solvent has an effect on cell morphology on collagen surface. Cells were rounded on collagen surface, when dodecafluoroheptanol was used. On the other hand, cell spreading was observed when cultured on collagen surface at the interface using perfluorohexane. The cells exhibited typical spreading on collagen surface in contact with the culture medium and also at the reverse side of the collagen surface in contact with perfluorohexane.

1. Introduction

Fluorous solvents have high-oxygen solubility [1]. They are biologically inert, highly dense liquids that can be used for culture considering the reported low cytotoxicity [2]. Giaever and Keese showed that murine fibroblast cells grow in the presence of fluorocarbons [3]. Liquid–liquid interface culture using fluorous solvents has also gained wide interest on accounts of ease of (a) collection of cells by pipetting and centrifugation, and (b) subcultivation by eliminating the use of harmful proteolytic enzymes [4]. Shiba et al. reported that it is possible to cultivate anchorage-dependent animal cells at the interface between the aqueous culture medium (upper layer) and the fluorous solvent (lower layer) [5]. Moreover, subcultivation of cells grown at the interface was possible without trypsin treatment.

Recently, we reported that mouse melanoma B16 cells thrived when cultivated at the interface between 2 kinds of liquids: DMEM-F12 culture medium and dodecafluoroheptanol [6]. Significantly, dissolved oxygen in the culture medium that was consumed by cells during culture via liquid–liquid interface system was replenished from the fluorous solvent. However, the cells were rounded at the liquid–liquid interface and were different, when they were grown on conventional solid substrate such as microcarrier beads and plastic. Spreading typical to anchorage-dependent B16 cells grown on polystyrene culture dishes was not observed. Although the anchorage-dependent cells could grow and spread on nonrigid surfaces such as gels and soft agar, we could surmise from the results that cell spreading did not occur because the fluorous solvent possibly lacked enough rigidity to serve as scaffold to support cell spreading.

Collagen is a structural protein at the extracellular matrix that supports cell adhesion [7]. This extracellular matrix protein does not only provide cells the anchorage but also influences cellular behavior, such as morphology, migration, and spreading. Ando et al. demonstrated that human endothelial cells spread and proliferated until they formed confluent monolayers, when cultured on liquid–liquid interface that was pretreated with extracellular matrix proteins, such as laminin, fibronectin, or collagen [8].

In this research, a collagen membrane was employed in liquid–liquid interface culture to serve as solid substrate, where cells could attach and spread. The behavior of various types of cells (mouse melanoma B16, Mardin Darby canine kidney (MDCK), mouse embryonic fibroblast 3T3, and human hepatocarcinoma HepG2) on the collagen membrane at the interface was investigated. Cell growth, attachment, and spreading on collagen surface in contact with either the aqueous culture medium layer, or the fluorous solvent layer were examined. Moreover, two different

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kinds of fluorous solvents, perfluorohexane (1,1,1,2,2,3,3,4,4,5,5,6,6,6-tetradecafluorohexane), and dodecafluoroheptanol (2,2,3,3,4,4,5,5,6,6,7,7-dodecafluoro-1-heptanol) were used to compare the effect on cell spreading during culture on collagen membrane. The liquid–liquid interface culture having a collagen membrane at the interface is being developed in this study in view of the potential application in cell and tissue engineering.

2. Results and discussion

2.1. Effect of dodecafluoroheptanol

To validate the assumption that cells need a rigid surface to attach and to spread, a collagen membrane was set at the junction between the aqueous culture medium and the fluorous solvent layer. Anchorage-dependent cells (B16, MDCK, 3T3, and HepG2) were each seeded to collagen membrane in 24-well plates and cultured on the surface in contact with the culture medium (upper layer) as shown in Scheme 1C. The utility of the collagen membrane for cell attachment and spreading during liquid–liquid interface culture was observed using dodecafluoroheptanol, a fluorous alcohol, as lower layer.

When cultured at the liquid–liquid interface in the absence of a solid substrate (Scheme 1B), cells did not spread as shown in Fig. 1A–D (Control). Surprisingly, similar morphology was obtained regardless of the presence of collagen membrane, when dodecafluoroheptanol was used. Cell spreading did not occur, even when cells were seeded and grown on a collagen membrane at the interface as shown in Fig. 1E–H. Although cells attached to the collagen membrane, cells remained rounded even after 3 days of culture. Moreover, black coloration of the culture medium was observed with B16 cells using dodecafluoroheptanol. Mouse melanoma B16 cells secrete the black pigment melanin [9]. Isolation of the black substance from the culture medium and analysis of the mass spectra obtained confirmed that the black pigment is eumelanin. Elevated production of melanin by melanocytes is evidence of stress caused by exposure to ultraviolet radiation or induced by the presence of chemicals such as heavy metals and oxidizing agents [10]. The results suggest that the fluorous alcohol used in this study inflicts stress to B16 cells resulting to elevated secretion of the black pigment melanin. Expectedly, black coloration of the culture medium was not observed, when cells such as MDCK and HepG2 that are not melanocytes were exposed to dodecafluoroheptanol.

Fluorine containing alcohols have acidic properties [11]. The pH of the culture medium was monitored to determine whether increased production of melanin was brought about by the acidity of the culture medium due to the presence of the fluorous alcohol. After 48 h incubation, the culture medium pH was 7.06 confirming that the buffering capacity of the incubator system (5% CO_2 supply) was sufficient to maintain the pH even in the presence of dodecafluoroheptanol. Hence, the elevated production of melanin was not caused by acidic insult to cells.

To ascertain if the cell behavior on collagen membrane was specific to the fluorous alcohol, further investigation was carried out using a different perfluorinated solvent.

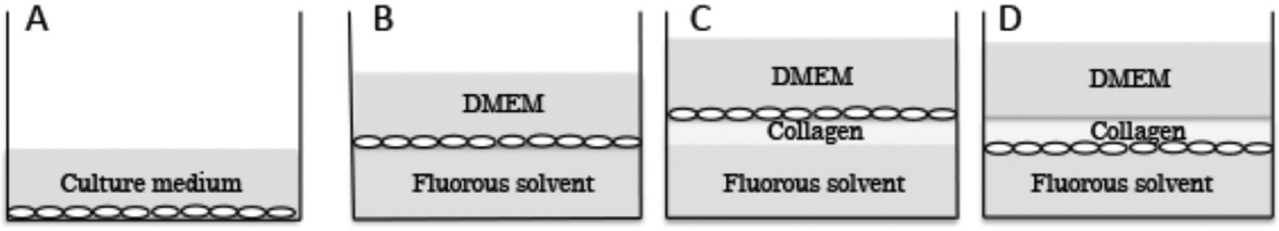
2.2. Effect of perfluorohexane

Initially, the utility of perfluorohexane for liquid–liquid interface system (without collagen membrane) was investigated. The culture medium was carefully overlaid on perfluorohexane and the cells were seeded at the interface as shown in Scheme 1B. After incubation for 72 h, results showed that the cells were alive indicating that perfluorohexane did not show cytotoxicity. The 3T3 cells and B16 cells formed cell aggregates and a cell sheet, respectively, at the interface as shown in Fig. 2E and H. However, when compared to cells grown on conventional polystyrene well plates (Fig. 2A–D), typical cell spreading did not occur (Fig. 2E–H). Cell morphology was similar to that observed when dodecafluoroheptanol was used for liquid–liquid interface culture in the absence of solid support [6].

To investigate whether the collagen membrane at the interface could support cell spreading by using perfluorohexane, the four kinds of cell lines were seeded and allowed to grow on the surface in contact with either the culture medium or the perfluorohexane layer as shown in Scheme 1C and 1D, respectively. After 72 h incubation, results showed cell adhesion and spreading to the collagen surface in contact with either liquid. Similar to cells grown on polystyrene well plates (Fig. 2A–D), the various cells used in this study were anchored and proliferated on collagen surface (Fig. 2I–P). Significantly, HepG2 cells that usually formed aggregates on polystyrene dishes exhibited a monolayer, when grown on collagen membrane. Moreover, the viability of all cell types grown on collagen membrane increased as compared with the cells grown in the absence of collagen substrate as shown on Table 1. The number of live cells almost doubled, when anchorage-dependent cells were cultured on collagen membrane. Generally, cell viability and number of live cells were higher for cell grown on collagen in contact with the culture medium that contains nutrients necessary for growth, when compared with cells grown on collagen at the fluorous solvent side.

2.3. Comparison between fluorous solvents

The results confirmed that the kind of fluorous solvent used has a significant effect on cell behavior on the collagen membrane at the interface. As mentioned above, cells adhered to but did not spread on collagen surface, when dodecafluoroheptanol was employed. On the other hand, when perfluorohexane was used, the cells adhered, spread, and formed a confluent monolayer such as that typically observed on polystyrene dishes.



Scheme 1. Culture of cells (A) on polystyrene well plate, (B) at the liquid–liquid interface, and (C and D) on collagen surface in contact with the culture medium or fluorous solvent, respectively.