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Short Communication

Cellular glycosylation of amphiphilic saccharide primer in liquid/liquid interface culture system employing fluorous solvents

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ABSTRACT

As fluorous compounds have extremely high oxygen-carrying capacity, liquid/liquid (culture medium/fluorous solvent) interface culture system is one of their attractive applications in the field of biotechnology. However, it remains to be elucidated whether the metabolism of the cells cultured in such a system could work properly. To determine whether glycolipid metabolism of these cells are affected by such a low adhesive condition at the interface, we evaluated cellular glycosylation of a synthetic pseudo-glycolipid by mouse melanoma B16 cells cultured in liquid/liquid interface system using three kinds of fluorous solvents. The results showed that the saccharide primer was sialylated by the cells when cultured at the interface using Fluorinert™ FC-72 or perfluorodecalin, but not with dodecafluoroheptanol. The results of this work confirmed that glycolipid metabolism of cells cultured at the interface of aqueous/fluorous culture system functioned properly as well as normal aqueous culture system but depending on the kind of fluorous solvent used. Ganglioside biosynthesis was not compromised amidst an unusual environment such as the absence of a solid substrate for cell attachment and the presence of a perfluorinated solvent.

1. Introduction

Fluorous solvents, *i.e.* highly fluorine-substituted liquid chemicals, have approximately 20 times higher O_2 solubility than water [1] because of their relatively weak intermolecular interactions. Hence, they can be used as flexible O_2 carriers to facilitate oxygen supply to cells or tissues [2]. As they are immiscible with water, several studies have addressed animal cell culture at the interface formed between fluorous solvent and overlying culture medium [3–6]. These studies have demonstrated that the anchorage-dependent cells were able to grow and proliferate at the interface. Such culture system is expected to enhance the oxygen access to the cells and to overcome the poor oxygen supply, which is often considered to be a limiting factor of three-dimensional cell culture *in vitro* [5]. In addition, this simple culture method does not require trypsin treatment for subcultivation, thereby the adverse effects by trypsin [3] can be eliminated.

In previous studies [7], we evaluated morphology, growth and oxygen consumption of the cells cultured on the liquid/liquid interface, and reported that 2,2,3,3,4,4,5,5,6,6,7,7,-dodecafluoro-1-heptanol (DF-heptanol) serves as an oxygen reservoir that could supply O_2 to the culture medium. However, there is also an intriguing issue of whether metabolism of these cells are affected by such a low adhesive condition at the interface. Although some studies have reported on metabolic response of cultured cells to liquid/liquid interface system employing oxygenated perfluorocarbons [8], little is known about the effects of the system itself on cellular metabolism.

In this research, we focused on oligosaccharide elongation, particularly biosynthesis of gangliosides by the cells at the liquid/liquid interface. Gangliosides are sialic acid-containing glycosphingolipids, which are known to exist in the lipid raft on the surface of the plasma membrane and to be associated with a variety of biological phenomena, such as cell adhesion, signal transduction, malignant alteration of cancer cells, pathology of diseases, etc [9,10]. Unlike nucleic acids and proteins, they are not directly encoded in DNA and they are susceptible to the physiological conditions of the cells. Therefore, in order to elucidate the gap between genomic sequences and complicated biological phenomena, it is important to explore ganglioside behavior, which reflects physiological changes of the cells.

Abbreviations: DF-heptanol, dodecafluoroheptanol; PFD, perfluorodecalin; TI-DF, DMEM/F12 containing Insulin-Transferrin-Selenium-X; HPTLC, high-performance thin layer chromatography.

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We evaluated biosynthesis of ganglioside GM3 by mouse melanoma B16 cells at the culture medium/fluorous solvent interface using saccharide primer method. This method is an effective way to monitor biosynthesis of oligosaccharides without destroying the cells, in which the cells are incubated with saccharide primer, a synthetic pseudo-glycolipid, and the primers are taken into the cells and subsequently glycosylated by cellular glycosyltransferase [11,12]. Using this approach, previously we assessed the effects of some factors of cell culture conditions, *e.g.* xenobiotics [13] and the passage number of the cells [14], on cellular glycosylation.

In this study, mouse melanoma B16 cells were cultured at the interface formed between fluorous solvents, such as DF-heptanol, Fluorinert™ FC-72 (commercially available mixture of perfluorochemicals whose main constituent is perfluorohexane [15]) or perfluorodecalin (PFD), and culture medium containing dodecyl β-lactoside (Lac-C12) primer that mimics lactosylceramide, a precursor of a variety of glycolipids. How the cells behave in the presence of the saccharide primer during liquid/liquid interface culture was investigated and the effect of different fluorous solvents was compared.

2. Results

2.1. Morphology and viability of B16 cells at the liquid/liquid interface

In order to investigate the effect of different fluorous solvents on cell morphology, B16 cells were observed using an optical microscope after 48 h of culture. Whereas the cells spread in polystyrene dish (Fig. 1A), the cells cultured in liquid/liquid system employing DF-heptanol remained rounded and existed in monolayer (Fig. 1B). As for the cells cultured with FC-72 or PFD, they were also rounded, but were grown as three-dimensional multicellular aggregates (Fig. 1C, D). From a macroscopic point of view (Fig. 1E), a sheet-like structure of the cells was observed when DF-heptanol was used. In addition, the medium overlying DF-heptanol was greatly blackened, which is considered to be due to melanin production.

The number of the viable cells after 48 h for each condition is shown in Fig. 2. When FC-72 or PFD was used, there was no significant difference from control. By contrast, with DF-heptanol, the number of the viable cells was lower than control, indicating that DF-heptanol may not be suitable for this study because of its cytotoxicity.

In the previous work using DF-heptanol as oxygen reservoir during biphasic culture [7], the cytotoxic effect of the fluorous solvent was not easily recognized because the medium used was supplemented with 10% fetal bovine serum (FBS) that promoted growth and proliferation. On the other hand, cell culture in the presence of the saccharide primer as scaffold for glycosylation of cellular enzymes in this work required the use of culture medium without FBS to avoid the serum from interfering with ganglioside accumulation. This condition limited cell growth and proliferation. In both studies, cells thrived but the adverse effect of DF-heptanol on cell viability and its repercussion on glycosylation became more evident when compared with other fluorous solvents such as FC-72 and perfluorodecalin.

2.2. HPTLC analysis and identification of the sialylated product

After incubation of B16 cells with Lac-C12 primer for 48 hours, the cells and the medium fractions were collected and analyzed by HPTLC. HPTLC results of the lipid extract from the cell homogenates and the culture medium are shown in Fig. 3A. The compounds containing sialic acid were visible as blue-violet bands [19]. In the presence of the Lac-C12 primer, there were new bands corresponding to a putative sialylated product, ganglioside GM3 analogue, in medium fraction when FC-72 or PFD was used (Lane 14, 15). Such a band could not be detected when DF-heptanol was used (Lane 13). It was shown that the primer was not taken into the cells cultured at medium/DF-heptanol interface (Lane 5).

In order to confirm the structure of the products, they were scraped from the HPTLC plate, extracted with MeOH, and then identified from ESI-MS and enzyme assay. Mass spectrum of the product showed a peak at m/z 800.1. Fragmentation of the product showed peaks at m/z 508.9 and 289.7, corresponding to Lac-C12 primer and *N*-acetylneuraminic acid (NeuAc), respectively. Treatment with α2,3-sialidase showed that the product was hydrolyzed (Fig. 3B). Hence, it was confirmed that the product has a terminal α2-3 sialic acid moiety. From these results, the product was identified as a GM3 (NeuAc(α2-3)Lac-C12) analog.

2.3. Effects of the different fluorous solvents on the amount of sialylated primer

The amounts of GM3 analog and endogenous GM3 in cell and medium fractions were quantitated by densitometric analysis (Fig. 4). The amounts of GM3 analog from the cells cultured in polystyrene flask, and liquid/liquid interface system using FC-72 and PFD were 11.87, 10.47 and 10.72 μmol/flask, respectively (SDs = 0.48, 1.80 and 1.76 μmol/flask, respectively). There were no significant differences among them.

3. Disucussion

The present study demonstrated that Lac-C12 primer was sialylated by B16 cells when cultured at the interface using perfluoroalkanes, such as FC-72 or PFD, in the same way as when

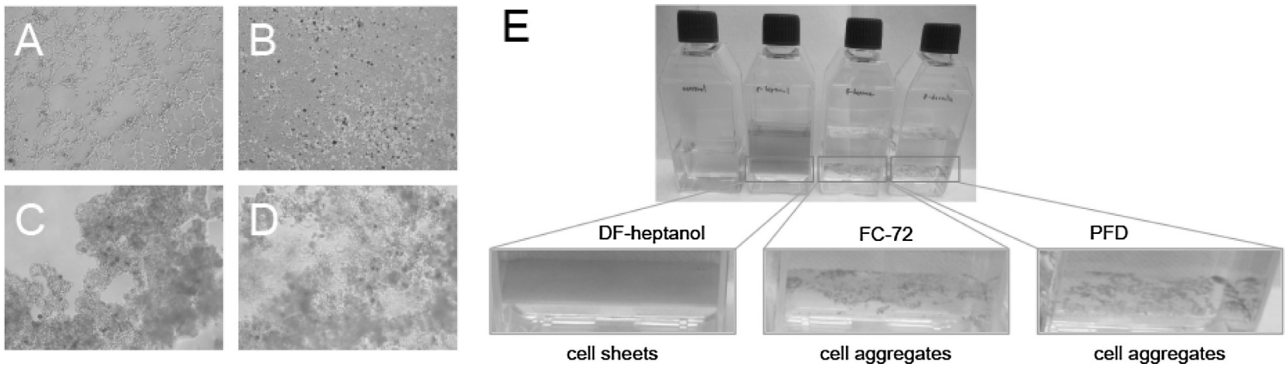

Fig. 1. Morphology of B16 cells during (A) normal culture in polystyrene dish, and liquid/liquid interface culture employing (B) DF-heptanol, (C) FC-72 and (D) PFD. (E) The examples of liquid/liquid interface culture system after 48 h of culture, From left to right: normal culture, liquid/liquid interface culture employing DF-heptanol, FC-72, PFD.