A novel tool for suspension culture of human induced pluripotent stem cells: Lysophospholipids as a cell aggregation regulator

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1. Introduction

A substantial number of hiPSCs (more than $10^9$ cells) will be necessary for cell therapy of various diseases, such as myocardial infarction [1], diabetes [2], and hepatic failure [3]. Suspension culture has attracted attention as a mass culture method for hiPSCs for not only in clinical trials but also in commercialization. However, the scalable and cost-effective culturing of high-quality hiPSCs and their derivatives, especially for clinical applications, remains a challenge. Suspension culture based on aggregates provide simplicity and a reduction in the number of processing steps required compared to adhesion culture at large scale culture or expansion culture.

Current reports using bioreactor for expansion of human pluripotent stem cells sometimes implement with the strategy of seeding with single cells suspension, which often forms aggregates with heterogeneous sizes. The size of aggregates greatly affects the state and quality of the subsequent cells, so controlling aggregate size is essential for the homogeneity, reproducibility, and efficiency of the desired process [4]. Excessive agglomeration of aggregates can lead to growth arrest, cell death, or uncontrolled spontaneous differentiation as well as human embryonic stem cells (hESCs) [5,6]. To avoid excessive agglomeration of aggregates and make their further growth, mechanically and hydrodynamically regulation have been attempted [7]. Such as impeller shearing easily prevents excess aggregation [8]. However, too high shear stress could affect cell viability and pluripotency of hiPSCs [7]. Therefore, the regulation of cell aggregation using unmechanical methodology is important for the establishment of versatile suspension culture systems.

Before, we reported a new biochemical approach for regulating the aggregation of hiPSCs by using lipids associated albumin in suspension culture [9], whereas, the lipids responsible for the suppressive effect of aggregation were unclear. In this report, we identified principal lipids regulating aggregation size of hiPSCs. This study aimed to develop a simple and robust method for the suspension culture of hiPSCs and suggested to be a breakthrough technology for the large-scale and cost-effective production of hiPSCs for regenerative medicine.
2. Materials and methods

2.1. Maintenance of human induced pluripotent stem cell lines

The hiPSCs line, TkDN4-M was provided by Centre for Stem Cell Biology and Regenerative Medicine, The University of Tokyo, Japan. The hiPSCs line, 201B7 was provided by Kyoto University, Japan. The hiPSCs line, RPChiPS771 was purchased from ReproCELL, Japan. TkDN4-M and 201B7 were cultured on truncated recombinant human vitronectin-coated dishes with Essential 8™ medium (both from Thermo Fischer Scientific). RPChiPS771 was cultured on truncated recombinant human vitronectin-coated dishes with StemFit AK02N (from Ajinomoto, Japan). For subculture, single cells were seeded with 10 μM Y-27632 (FUJIFILM Wako Pure Chemical Corporation, Japan) in the medium. The initial seeding was fixed at a viable cell density of 1 × 10⁴ cells/cm². Cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂, and the medium was changed every day with fresh medium without Y-27632. On day 4, cells were subcultured as described below. Cells were treated with Accutase (from Innovative Cell Technologies) for 4 min incubation at 37 °C, and the hiPSCs were dissociated into single cells by pipetting with fresh medium containing 10 μM Y-27632. After centrifugation, the supernatant was discarded, and cells were re-suspended in fresh medium with 10 μM Y-27632. Viable cells were counted on a hemocytometer with the trypan blue exclusion method, and cells were re-seeded in a new culture dish.

2.2. Aggregation assay

The method for aggregation assay to detect the lipid that acts as a suppressor of aggregation describes in Fig. 1 briefly. hiPSCs cultured on truncated recombinant human vitronectin-coated dishes were dissociated into single cells by soaking for 3–5 min in Accutase and suspended in medium containing 10 μM Y-27632. The cell density of the collected single hiPSCs suspension was calculated by cell counting with trypan blue staining. Then, 1.3 mL of 2 × 10⁵ cells/mL cell suspension in fresh medium containing 2 mg/mL BSA and 10 μM Y-27632 was seeded into a flat-bottom 12-well plate (Sumilon Multi-well plate, Sumitomo Bakelite Co, Ltd, Japan). After inoculation, candidate lipids were added to the culture medium and the cells were then incubated for 1 day on a rotary shaker (OS-762, Optima, Japan) with shaking at 90 rpm. Subsequently, the medium was replaced with culture medium without the lipids and additional medium changes were performed every day.

2.3. Suspension culture with lysophospholipids

To investigate the ability of cell proliferation and pluripotency, hiPSCs were cultured continuously after the formation of cell aggregates. After dissociating the hiPSCs colony into single cells, 2 × 10⁵ cells/ml were seeded with 10 μM Y-27632 and 2 mg/mL BSA into a flat-bottom 12-well plate (1.3 mL per well) (Sumilon Multi-well plate, Sumitomo Bakelite Co, Ltd, Japan). After inoculation, LPA or S1P were added to the culture medium to yield a final concentration of 0.2 μg/mL and the cells were then incubated for 2 days on a rotary shaker (OS-762, Optima, Japan) with shaking at 90 rpm. Subsequently, the medium was replaced with culture medium without the lipids and additional medium changes were performed every day.

2.4. Glucose consumption measurement and cell counting

Medium samples were collected during the daily medium changes after day two of incubation. Glucose consumption was measured using a biosensor BF-5iD (Oji-Keisoku Kiki, Japan). Aggregates were collected from suspension cultures on day five. Collected aggregates were dissociated into single cells by incubating for 10 min in Accutase and pipetting. Live cells were counted using the trypan blue exclusion test. P-values were determined by a two-tailed paired Student’s t-test from comparison with the cell number of BSA condition and cell number of LPA or S1P condition.

2.5. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Collected cells were dissolved in TRIzol reagent (Thermo Fisher Scientific). Total RNA samples extracted and purified using the TRIzol method and PureLink RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. The cDNA was

![Fig. 1. Schematic illustrations of aggregation assay for detection of aggregation inhibitor.](Image)
Table 1
List of primers used for quantitative reverse transcription–polymerase chain reaction (qRT-PCR).

| Gene       | Forward primer (5’-3’) | Reverse primer (5’-3’) |
|------------|------------------------|------------------------|
| b-Actin    | CCTCATGAAATCTCTACCGCA  | TTGCAATGTGATACCTTGGA  |
| OCT4       | ACGGGTACCATGATGAAAGTG  | CTCCTCATCTGCTGTGTTCA  |
| SOX2       | CACCAATCCATCATCACACTAC| GCAAAGCTTCTCCGCTTACCA |
| Nanog      | AGCCCTCCAGCAGATGAAAGTC| CTTCGCAACATGAGAAGCTCA |
| KLF4       | AAATGCAAGGACATACCAGGA  | CCCTACTGCTGCTGTGTTCA  |
| LPA-R1     | ACCAGCTCTCCTTCAACAGG   | AATGACCTGAAACCCAAGAG  |
| LPA-R2     | CTCGCTCGTCCATCTCCTCTC| ACTCCGATCTCAACAGTCTC |
| LPA-R3     | AATTGGCTCTGCAACATCTC  | ACAGCTCAGTCAACAGTCTC |
| LPA-R4     | CTCTTGGCTCCATCTCCTCTC| CTCCTGCAACATGAGAAGCTC|
| LPA-R5     | ACAGCTCCTCAACAGGACTTT | GTTGGCCAGTCTCCGTTAGG |
| LPA-R6     | CTTCTGCGTCTCTCAACAGG  | AAGAACAGGATGTTCTCCGTA |
| STP-R1     | TGCTTCTGCTTCCATCTGTC  | CTTACGCTTGCAACAGGAC  |
| STP-R2     | GTGGCCATTCCCAAGGCCA   | GCAGTAGATCGCCAACAGAC |
| STP-R3     | GGTCAATCCCAAGGCCGCTA  | ACTTGGACACAGCCAACAGA |
| STP-R4     | CTCTACTCCAAGGCTTACC   | AAGAACAGGAGTATCAGAG |
| STP-R5     | GGCTACTTGTGACATAGCAA  | CTAATATGTGATTGTGTTG |
| LPI-R      | TCCATTGTGTTTTCTCCAAGCT| GAAGGCAGATATCCGTTACG |

b-Actin, actin beta; OCT4, POU class 5 homeobox 1; SOX2, SRY-box 2; Nanog, Nanog homeobox; KLF4, Kruppel like factor 4; LPA-R, lysophosphatidic acid receptor; STP-R, sphingosine-1-phosphate receptor; LPI-R, lysophosphatidylinositol receptor.

2.6. Flow cytometry

For flow cytometric analyses, the cultured aggregates were dissociated into single cells with TrypLE select and fixed with 4% PFA for 20 min at room temperature. Fixed cells were washed with phosphate buffered saline (PBS) containing 3% fetal bovine serum (FBS). To permeabilize for staining to intracellular protein markers, cells were treated in methanol on ice overnight. After blocking with 3% FBS/PBS, the cells were stained with antibodies; anti-OCT4-PE (653704, Biolegend, USA) and anti-Nanog-Alexa Fluor® 647 (674010, Biolegend, USA). These samples were compared to the samples of cells stained with isotype controls; mouse IgG2b-PE (400314, Biolegend, USA), mouse IgG1-Alexa Fluor® 647 (400136, Biolegend, USA). Flow cytometry data were acquired using a Guava® EasyCyte HT flow cytometer (Merck Millipore, USA) and analyzed using Guava®-InCyte software (Merck Millipore, USA).

2.7. Statistical analyses

Data are expressed as the mean ± SEM. For comparisons of discrete data sets, unpaired Student’s t-tests were used. Two-tailed P < 0.05 was considered significant.

3. Results

3.1. Inhibition of initial aggregation of hiPSCs by lysophospholipids

Our group reported that albumin-associated lipids prevented excess aggregation of hiPSCs [9]. Therefore, we searched for lipids with an ability to suppress aggregation. To examine the ability to inhibit aggregation of hiPSCs, we used aggregation assay based on swirling suspension culture (Fig. 1). Albumin is known to act as a transporter for fatty acids, cholesterol, lysophospholipids, and so on in the blood [11–14]. Under conditions such that large aggregates were formed when only lipid-free BSA (BSA) was added (Fig. 2A), synthesized using the ReverTra Ace qPCR RT Master Mix (Toyobo, Japan). qRT-PCR was performed on QuantStudio™ 7 Flex (Thermo Fisher Scientific) using KOD SYBR® qPCR Mix (Toyobo, Japan). β-Actin was used as the housekeeping gene for relative quantification of target gene expression. Primers are listed in Table 1. PCR cycling began with template denaturation and hot start at 98 °C for 2 min, then 5 cycles of 98 °C for 10 s and 68 °C for 30 s, 40 cycles of 98 °C for 10 s and 60 °C for 10 s and 68 °C for 30 s performed on a QuantStudio™ 7 Flex System with data collected during each cycle at the extension step. The qRT-PCR results were imported into Microsoft Excel and the average value of duplicate Ct values using the 2DDCt method.

![Fig. 2. Aggregation assay treated with different reagent.](image-url)
when Chemically Defined Lipid Concentrate (CDLC, Thermo Fisher Scientific Inc.) which was a mixture containing fatty acid and cholesterol was added with BSA, large aggregates were formed (Fig. 2C), whereas when LPA or S1P, known as lysophospholipids, were added with BSA, a plurality of small clumps were formed as well as lipid-rich BSA (AlbuMAX™) (Fig. 2B, 2D, and 2E). Even though LPA with different acyl groups was used, cell aggregation was suppressed (Fig. 3A and 3B). On the other hand, LPC, PA, LPI didn’t act as the suppressor of aggregation (Fig. 3C, 3D, and 3E).

To investigate the correlation between lipids concentration and aggregate size, aggregation assay was performed by adding various concentrations of lipids (Fig. 4). Box plots of aggregate diameters treated with various concentrations of LPA or S1P are shown in Fig. 4C and 4D. According to the size measurements, 20 µg/mL LPA resulted in regulated low diameter (39.9 ± 8.0 µm), whereas the lower concentration of LPA showed a larger size aggregate. For example, with 0.16 µg/mL LPA treatment, the aggregate sizes were more than 100 µm (124.9 ± 26.5 µm). These results indicate that the aggregate size increased with lower LPA or S1P concentrations.

To examine whether LPA has an effect of aggregation suppressing in other strains or other culture media with a higher concentration of LPA to consider the difference between the medium or the cell line, aggregation assay was carried out using 201B7 with Essential 8™ and RPChiPS771 with StemFit® AK02N. As a result, in both cell lines as well, LPA suppressed cell aggregation (Fig. 5). These results indicate that lysophospholipids, particularly LPA and S1P, suppress aggregation of hiPSCs.

### 3.2. Glucose consumption and cell yields

To investigate cell proliferation and pluripotency, cultures were continued in floating, following formation of aggregates under each condition (only 2 mg/mL BSA, 2 mg/mL BSA with 0.2 µg/mL LPA, or 5 mg/mL BSA with 0.2 µg/mL S1P) (Fig. 6). Day 1 after inoculation, the aggregates of more than 1 mm diameter were formed with only BSA (Fig. 6A), whereas the aggregates of around 100 µm diameter were formed with LPA or S1P (Fig. 6B and 6C). After treatment with LPA or S1P, excess aggregation was not observed until day 5 (Fig. 6E and 6F). As a result of measurement of the glucose concentration, the consumption of glucose in the aggregates formed under the addition of only BSA was saturated after the third day (Fig. 6G). On the other hand, when LPA or S1P was further added, glucose consumption increased continuously for 5 days (Fig. 6G). Since the cell yield under the LPA or S1P condition was higher than the one under the only BSA condition, it showed that cell proliferation was improved by LPA or S1P treatment because the size of aggregates was suitable for proliferation (Fig. 6H).

### 3.3. Expression of pluripotent markers

To confirm the pluripotency of hiPSCs cultured with the lipids, we examined qRT-PCR and FACS from samples of collected cells. qRT-PCR analyses revealed that the cell aggregates formed with LPA or S1P expressed pluripotency genes at the same level or

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**Fig. 3. Aggregation assay treated with different lipids.** Cells (TkDN4-M) were cultured in Essential 8™ medium containing 2 mg/mL BSA supplemented with 0.2 µg/mL 1-Oleoyl LPA (A), 0.2 µg/mL 1-Palmitoyl LPA (B), 0.2 µg/mL 1-Oleoyl LPC (C), 0.2 µg/mL PA (D) or 0.2 µg/mL LPI (E) for 1 day on a rotary shaker with shaking at 90 rpm. (F)–(J) show the chemical structure of each lipid. The white scale bar is 500 µm.
Fig. 4. Morphologies and sizes of cultured aggregates after treatment with different concentrations of LPA or S1P. (A, B) Cells (TkDN4-M) were cultured in Essential 8™ medium containing 2 mg/mL BSA supplemented with different concentrations of LPA or S1P from 0.032 to 100 μg/mL for 1 day on a rotary shaker. The white scale bar is 500 μm. (C, D) Box plots show aggregate diameters obtained by randomly 30 aggregates treated with various concentrations of LPA or S1P from 0.16 to 100 μg/mL for 1 day on a rotary shaker with shaking at 90 rpm.

Fig. 5. Aggregation assay using different hiPSCs lines and different medium with LPA. 201B7 cells were cultured in Essential 8™ medium containing 2 mg/mL BSA supplemented with 0 μg/mL LPA (A) or 0.5 μg/mL LPA for 1 day on a rotary shaker (B). RPChiPS771 cells were cultured in StemFit® AK02N medium containing 2 mg/mL BSA supplemented with 0 μg/mL LPA (C) or 0.5 μg/mL LPA (D) for 1 day on a rotary shaker with shaking at 83 rpm. The white scale bar is 200 μm.
more as compared with hiPSCs cultured under two-dimensional (2D) adhesion condition (Fig. 6I and 6J). Furthermore, FACS analyses revealed that over 90% of the cell population in aggregates formed with LPA or S1P expressed OCT4 or Nanog (Fig. 6M, 6N, 6Q, 6R) as well as 2D culture, whereas, the cell population cultured with only BSA decreased OCT4 positive cells compared with 2D. These results demonstrated that the cell aggregates with the lipids kept the high-expression rates of pluripotent markers, similar to the rates observed for hiPSCs cultured under 2D condition.

3.4. Gene expression of receptors of lysophospholipids in hiPSCs

LPA and S1P are endogenous bioactive lipids which mediate a variety of biological cell responses such as cell proliferation, migration, differentiation, and apoptosis [15]. Their actions are mediated by binding to the G-protein-coupled endothelial differentiation gene (Edg) receptor subfamily, referred to as LPA-receptor 1–6 and S1P-receptor 1–5 [15]. Therefore, we analyzed the expression of these receptors in hiPSCs. qRT-PCR revealed that all receptors were expressed in hiPSCs (Fig. 7). Expression levels of S1P receptor 2, S1P receptor 4, and LPI receptor were lower than other receptors.

4. Discussion

In the present study, we demonstrated that lysophosphatidic acid (LPA) and Sphingosine-1-phosphate (S1P), known as lysophospholipids acting as a signaling molecule, suppressed aggregation of hiPSCs in suspension. The regulation of cell aggregation is important for effective proliferation in suspension culture. Actually, huge aggregates were hardly able to grow (Fig. 6D and E). The reason might be an insufficient supply of oxygen, nutrients, and growth factors such as basic fibroblast growth factor (bFGF) for the center cells of the aggregates [16,17]. On the other hand, it is possible that suitable size aggregates tend to promote not only cell growth but also pluripotency, which is supported by our glucose consumption and qRT-PCR analyses (Fig. 6D–G). S1P acts as a factor for the maintenance of human embryonic stem cells in an undifferentiated state via its receptors [18]. In this study, the receptor gene of LPA or S1P expressed in hiPSCs (Fig. 7). Therefore, lysophospholipids signaling may accelerate the pluripotency of hiPSCs aggregates.

Lysophospholipids such as LPA and S1P are known to perform various functions on cells via these receptors which have a potential of G protein-coupled receptor (GPCR) [19]. These GPCRs activate several signaling molecules such as RHO, PI3K, PLC, RAS, and AC (Fig. 8) [19]. LPA and S1P have been reported to be involved
in intercellular adhesion and migration in various cells [20,21]. Addition of LPA to cultures of cancer cells promotes migration while suppressing focal adhesions and adherens junction mediated by RHO/ROCK (Rho-associated kinase) signaling [20,22]. In adherens junction, E-cadherin is a key component of cell-cell adhesion and plays an important role in the undifferentiated state of iPSCs [23]. Therefore, it is possible that LPA and S1P bind to the receptor, and activate RHO/Rock signaling, and indirectly control cell-cell adhesion of aggregation. On the other hand, the ability of other lysophospholipids (such as LPI and LPC) to suppress cell aggregation was not observed. It predicts the reason why the expression level of the receptors for LPI or LPC was low level (Fig. 7) and RHO was not sufficiently activated by these lysophospholipids in hiPSCs.

Controlling the size of aggregates is important not only to maintain the proliferative potential and pluripotency (Fig. 6) [24] but also to induce differentiated cells from hiPSCs effectively for clinical use of cell therapy and regenerative medicine [25–30]. If the initial aggregates size of embryoid bodies is 150 μm, it is reported that the destiny leads to the mesoderm [28]. Although it is possible to create aggregates with a micro-fabricated microwell by controlling the size on the lab scale, mass culture requires a large area, which is difficult to manufacture cells for regenerative medicine on the commercial scale. It is preferable to use bioreactors already on sales such as Erlenmeyer flask and culture bag on the viewpoint of cost and versatility. In our unpublished study, we have confirmed that it can be grown in suspension culture of hiPSCs using Erlenmeyer flask, culture bags, and spinner flasks.

Fig. 6. (continued).
conventionally used for floating culture of mammalian cells such as CHO cells. Also, it is ideal for simplifying the manufacturing process that differentiation culture can be operated in the same system as it is.

5. Conclusion

We demonstrated that LPA and S1P prevented aggregation of hiPSCs in suspension culture. The result indicated that hiPSCs aggregate formed with these lipids could proliferate and keep the pluripotency. In conclusion, LPA and S1P regulate the aggregation size of hiPSCs. These lipids would contribute to simplify and generalize the suspension culture of pluripotent stem cells and help to realize regenerative medicine at an early stage.

Conflicts of interest

The authors declare that they have no conflict of interest.

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