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Differentiation function of embryonic stem cell inducible bio-interface patterned by photo-reactive phospholipid polymer

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Embryonic stem (ES) cells are well known for their differantiative totipotency and expected promising materials for the next generational regenerative medicine. But the differantiative mechanism of ES cells has been unexplained. The purpose of this study is to prepare a new ES cell culture system, which can maintain ES cells undifferentiated state by controlling ES cell colony size. For this purpose, we design noncytotoxic, cell-adhesion controllable and easily pattern formable polymer material. In this study, we prepared the micro-pattern surface by using with photo-reactive phospholipid polymer to regulate the growing scale of ES cells. This photo-reactive polymer was synthesized by a coupling reaction involving copolymer consisting of 2-methacryloyloxyethyl phosphorylcholine and methacrylic acid with 4-azidoaniline. We cultured mouse ES cells (129/Sv) on cell adhesion selective micro-patterned well made of photo-reactive phospholipid polymer. By alkaline phosphatase staining analysis against confluent ES cells in each well, ES cells cultured in micro-patterned well dyed much better than normal TCPS-cultured ES cells. This result indicated that the colony size of ES cells related to their differentiation state. We could control differentiation function of ES cells by controlling cell culture area in micro order.

Key words: embryonic stem (ES) cell, photo-reactive phospholipid polymer, micro-patterned surface, differantiation

1.Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of blastocyst-stage embryos [1,2,3]. ES cells are well known for their differantiative totipotency and expected promising materials for the next generational regenerative medicine combined with scaffolds studied in the tissue engineering field. Many researches have been made to induce differentiation of ES cells to various intended cell lines [4,5,6]. But the mechanism of differantiation of ES cells has not completely explained. It was already revealed that a cytokine, named leukemia inhibitory factor (LIF) is important biosignal to maintain the undifferentiated state of ES cells. However, the ES cells spontaneously aggregate and form colonies during the culture, and they were easily lost the undifferentiated state when the colony size was over the optimal size. Namely, the differentiation character of ES cells were affected not only biomolecules such as LIF, but also the growing area.

In this study, we prepared the micro-patterned surface using by photo-reactive phospholipid polymer to reveal the optimal size of colony for maintaining the undifferentiated state of ES cells. For this purpose, we need noncytotoxic, cell-adhesion controllable and easily pattern formable polymer material. We prepared photo-reactive phospholipid polymer made up of cell adhesion inhibitable 2-methacryloyloxyethyl phosphorylcholine (MPC) unit, methacrylic acid (MAc) unit and photo-reactive 4-azidoaniline methacrylate unit (AzMAc) and referred to as AzPMAc [7,8]. We made 3 different sizes of cell adhesion selective micro-patterned well made of AzPMAc to determine the effect of colony size against ES cell differentiation state. We cultured mouse ES cells (129/Sv) on this cell adhesion selective micro-patterned well until they become confluent growth condition in each well. By alkaline phosphatase (ALP) staining against confluent ES cells, we analyzed their differentiation state comparing with normal TCPS-cultured ES cells as a control.

2.Experiments

2.1 Preparation of photo-reactive phospholipid polymer AzPMAc

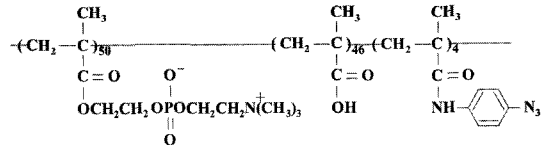


Fig.1 Chemical structure of AzPMAc

MPC copolymer consisting of MPC (50mol%) and MAc (50mol%) was obtained from NOF Co.Ltd. (Tokyo,Japan), and is referred to as PMAc. 4-Azidoaniline hydrochloride (90mg) and water soluble carbodiimide (WSC) (126mg) were dissolved in 2mL of PMAc solution (5wt%) and 98mL of pure water was added to the solution. The solution was stirred at room temperature under shading condition for 24h. To

remove unreacted 4-azidoaniline hydrochloride and WSC, the reaction mixture was dialyzed in 50 times amount of pure water under shading condition for 48 h. After dialysis, AzPMAc solution was freeze-dried to purify for 48h in shading condition. To determine molar concentration of azidophenyl group in copolymer, AzPMAc was analyzed by UV-VIS spectroscope (UV: V-560, JASCO).

2.2 Surface modification by AzPMAc

Under shading condition, 20μL of 2.0wt% AzPMAc aqueous solution was cast on 22ϕmm polyethylene (PE) plate and air-dried. These plates were UV irradiated (Spot-Cure; USHIO) through micro-patterned photomask. We used three different sizes of masks to determine correlations between the colony size of ES cells and their differentiation state (Table1). When an unpatterned surface was prepared, the photomask was not employed. After UV irradiation, PE plates were repeatedly rinsed with pure water to remove UV non-irradiated AzPMAc polymer from PE plates and air-dried again.

Table 1 Sizes of photomasks

Abb.	100μm/side	200μm/side	300μm/side
side of square (μm)	100	200	300
distance of each square (μm)	150	300	450

Surface analyses of AzPMAc modified and unpatterned PE plate were as following.

The surface chemical composition of plate was determined by X-ray photoelectron spectroscope (XPS) (AXIS HSi; Kratos). Survey scans (0-1100ev) were performed to identify the C, O, N and P elements. A take off angle of the photoelectrons was 90°. All binding energies were referenced the C_{1s} peak at 285.0ev.

The water static contact angles of bare PE plate and AzPMAc modified (unpatterned) plate were measured using a goniometer at room temperature. (CA-W: FACE). Water droplets of 6μL were contacted onto the plates and contact angles at 10s were directly measured by photographic images.

The formation of micro-patterned well was observed by phase-contrast microscopy.

2.3 Analysis of mouse ES cells differentiation

On the AzPMAc micro-patterned well (100, 200, 300μm/side), we cultured mouse ES cells according to the established ES cell culture protocol [9].

Primary mouse embryonic fibroblast (PMEF) cells were seeded $5x10^4$ cells/cm^2 on 10% gelatin coated 100ϕmm dish and incubated at 37°C with 5%CO_2 humidified air until becoming confluent on the dish. The PMEF media contained DMEM + 10%FBS. After becoming confluent, PMEF cells were treated with Opti-MEM + mitomycin C (MMC) 10μg/mL for 2h to inactivate, and transfered $5x10^4$ cells/cm^2 to the micro-patterned well on 22ϕmm PE plate. The micro-patterned plates were put into 6-well plate. The next day, micro-patterned plates were rinsed by phosphate-buffered saline (PBS) to remove non-adhered PMEF cells. On MMC treated PMEF feeder layer, mouse ES cells (129/SV) were seeded $2x10^4$ cells/cm^2 and incubated at 37°C with 5% v/v CO_2 humidified air until becoming confluent on the micro-patterned well. The ES media was containing KO-DMEM + 15wt%KSR + LIF 1000U/mL + 2-Mercaptoethanol 0.1mM + 1wt%L–Glutamine + 1%NEAA and changed daily. ES cells were monitored microscopically their formation of colonies. After becoming confluent in the micro-patterned well, ES cells were fixed by 3.8% formalin in PBS buffer and stained using alkaline phosphatase (ALP) staining kit (Vector) to analyze their differentiation state. This kit was used according to manufactures instruction for ALP staining. ALP is a hydrolase enzyme marker for ES cell differentiation; undifferentiated ES cells expressed high level of membrane ALP and differentiated ES cells expressed low level of ALP. When ES cells stained their expression of ALP, undifferentiated ES cells should show brighter red coloration than differentiated ES cells.

3.Results and Discussions

3.1 Preparation of photo-reactive phospholipid polymer AzPMAc

UV absorbance peak shift from 4-azidoaniline hydrochloride (aq) to AzPMAc (aq) was observed by UV spectroscopy. This peak shift came from peptide bond between azidophenyl group and carboxylic group in AzPMAc. Also the amount of introduced azidophenyl group in AzPMAc was estimated 4% by UV spectroscopy. The chemical composition (mol%) of AzPMAc was MPC/MAc/AzMAc = 50/46/4.

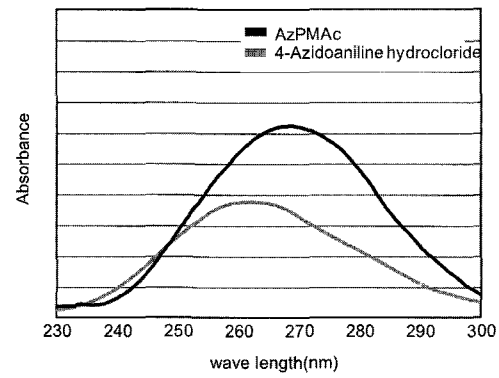


Fig.2 UV absorbance spectra of AzPMAc, and 4-azidoaniline hydrochloride solved in water

3.2 Surface modification by AzPMAc

Bare PE plate and AzPMAc modified plate were analyzed to confirm the surface modification.

Bare PE plate and AzPMAc modified plate were analyzed by XPS. Only AzPMAc modified plate showed the peaks of the nitrogen atom region (N_{1s}) at 403eV and the phosphorus atom region (P_{2p}) at 133eV. Those peaks were specific to the phosphorylcholine group in the MPC unit.

Water static contact angle of AzPMAc modified plate was lower than that of bare PE plate (bare PE plate;

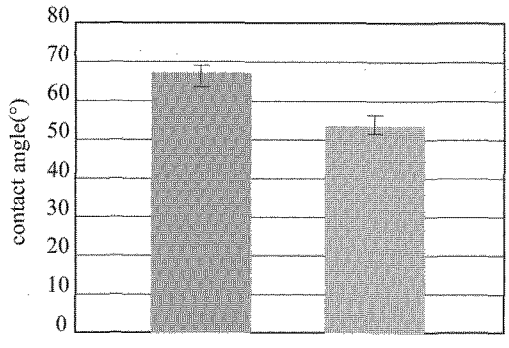
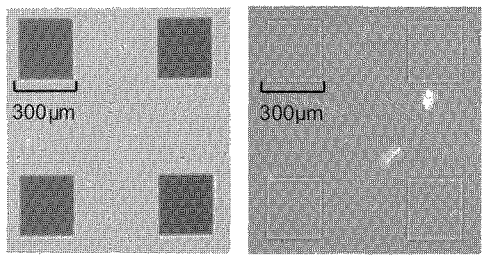


Fig.3 Water static contact angle

67.2°, AzPMAc modified plate; 52.5°) (Fig.3). This result indicated that MPC unit in AzPMAc increased surface hydrophilicity of AzPMAc modified plate.

The AzPMAc coated surface was UV irradited with a photomask in which dark squares are photo impermeable area (Fig.4(a)). (We showed only the result of 300μm/side case.) After UV irradiation, clear micro-patterns of AzPMAc gel were observed by phase-contrast microscopy (Fig.4(b)). The surface patterns were the same as those of the photomask. Insides of the square were cell adherable PE area and outsides were cell adhesion inhibitable AzPMAc gel area. In this case each squares were 300μm/side.

These MPC unit derived properties showed successful surface modification of PE plate by AzPMAc.



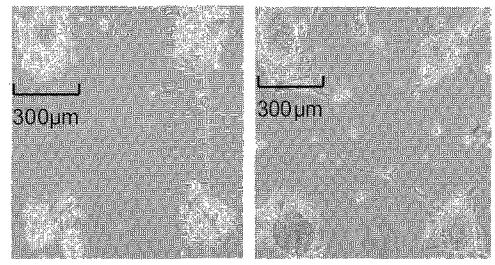
(a) photomask (b)AzPMAc well

Fig.4 Images of micro patterned well

Every well was 300μm/side. In Fig.4(a), black squares were photo impermeable area. In Fig.4(b), inside of squares were cell adhesive bare PE surface, and outside of squares were cell non-adhesive AzPMAc gel.

3.3 Analysis of mouse ES cells differentiation

Fig.5(a) is the image of ES cells 1 day after seeding on the micro-patterned wells by phase-contrast microscope. AzPMAc could inhibit cell adhesion, and clear cell patterns were observed. Fig.5(b) is the micrograph of ES cells 5 days after seeding. Colony formation of ES cells in the micro-patterned wells was observed. After becoming confluent in the micro-patterned well (6 days after cell seeding), we operated ALP staining test to the ES cells to analyze their differentiation state.



(a) ES day1 (b) ES day5

Fig.5 Images of ES cells cultured in micro-patterned well

Every well was 300μm/side. ES cells had selectively adhered inside of AzPMAc micro-patterned well and grown on PMEF cell feeder layer.

Micro-patterned ES cells showed brighter red coloration came from their strong expression of ALP than normal TCPS-cultured ES cells as a control. ALP staining result indicated that the differentiation state of ES cells cultured in micro-patterned wells differed from that of ES cells cultured in TCPS. Colony size regulated ES cells cultured in micro-patterned wells remained undifferentiated.

We showed only the result of 300μm/side case. But also the results of 100 and 200μm/side case showed ES cells cultured in micro-patterned wells remained undifferentiated.

4.Conclusions

Photo-reactive phospholipid polymer AzPMAc was successfully prepared. The micro-pattern surface could be prepared by using with photo-reactive AzPMAc. The size of micro-pattern can be regulated by photo-lithography technique. The AzPMAc modified area can inhibit the cell adhesion due to the phospholipid polymer can inhibit the protein adsorption which induce the cell adhesion. It inhibited cell adhesion. Using this AzPMAc, we could culture mouse ES cells on suitable patterns. On AzPMAc micro-patterned well ES cells formed colonies complied with well size. The result of ALP staining analysis against confluent ES cells indicated that the colony size of ES cells related to their differentiation state. In this study, ES cells cultured under 300μm/side micro-patterned well kept undifferentiated. In other words, ES cells, whose colony size were under 300ϕμm kept undifferentiated. We could control differentiation function of ES cells by controlling cell culture area in micro order.

5.References

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