A round-bottom 96-well polystyrene plate coated with 2-methacryloyloxyethyl phosphorylcholine as an effective tool for embryoid body formation

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Abstract

In this study, we proposed a culture method for forming embryoid bodies (EBs) from mouse embryonic stem (ES) cells using a round-bottom 96-well polystyrene plate coated with 2-methacryloyloxyethyl phosphorylcholine (MPC plate). MPC is a phospholipid biocompatible polymer and prevents cells from adhering to the culture surface. The ES cells were seeded at 1000 cells per well in the MPC plate with 200 μ l of medium. After 5 days of static incubation, a spherical cell aggregate termed EB was formed in a well. The size (diameter) of resulting EB was approximately 550 μ m and it contained approx. 22,000 cells. It seems that the non-adhesiveness and the roundness of the well are important factors to form a good EB. Transferring the EBs to the attached differentiation culture, the EBs spread out and flattened, and the beating cells (cardiomyocytes) were effectively generated in the outgrowth of EBs. The round-bottom 96-well polystyrene plate coated with MPC is an effective tool for EB formation.

Introduction

Embryonic stem (ES) cells are totipotent lines derived from the inner cell mass of developing blastocysts (Evans and Kaufman 1981; Martin 1981). Mouse ES cell lines have been widely used in the field of mammalian developmental biology because they retain their totipotential capacity and are able to generate cells of all lineages, including the germ line, after being introduced into host blastocysts (Bradley et al. 1984). Moreover, ES cells are capable of unlimited undifferentiated proliferation *in vitro*, and are able to spontaneously differentiate and to generate a wide variety of cell types. Therefore, ES cells are expected as a

renewable source of cells for cell-based therapy. For *in vitro* studies, mouse ES cells can be maintained on a feeder layer of cells and/or in the presence of leukemia inhibitory factor (LIF) to be maintained their undifferentiated state.

There are many ways to allow ES cells to differentiate spontaneously *in vitro*. The basic scenario for inducing differentiation is to form three-dimensional cell aggregates termed embryoid bodies (EBs). To perform *in vitro* differentiation, simply remove anti-differentiation factors such as feeder cells and LIF from the culture medium. This results in heterogeneous collection of EBs. Therefore, a more controlled culture system for EB formation is required. Various culture systems

have been used for the formation of EBs *in vitro* (Keller 1995). These include liquid suspension (LS) culture (Dang et al. 2002), methyl cellulose (MC) culture (Wiles 1993), and hanging drop (HD) culture (Shamblott et al. 2002). Under these culture conditions, the ES cells are unable to adhere to the surface of the culture plate, and generate spherical aggregates of differentiated cells as EBs. The choice of method to be used depends largely on the desired outcome. The HD culture is frequently used for the EB formation. Hanging droplet gives ES cells a good environment for EB formation. There is no place for cell adhesion in the hanging droplet. The round form of the droplet helps the aggregation of the cells.

A phospholipid polymer containing a 2-methacryloyloxyethyl phosphorylcholine (MPC) moiety is a biocompatible polymer. On the MPC copolymer surface, protein adsorption was reduced (Ueda et al. 1991; Ishihara and Iwasaki 1998) and cell adhesion was prevented (Zhu et al. 2002; Watanabe and Ishihara 2003). It is supposed that nonadherent environment facilitates cell aggregation. For example, the formation of multicellular aggregates of hepatocytes has been frequently performed in a nonadherent environment (Koide et al. 1990; Kurosawa et al. 2003a). Therefore, a round-bottom culture plate coated with MPC must be suitable for the EB formation.

This study is an attempt to assess the efficiency of using the round-bottom culture plate coated with MPC for forming EBs from mouse ES cells.

Materials and methods

Multi-well plates for EB formation culture

EB formation was performed using various round-bottom (U-bottom) 96-well plates. These were polystyrene (PS) plates (Nunc, No. 163320), 2-methacryloyloxyethyl phosphorylcholine (NOF Co. Japan) coated polystyrene (MPC) plates (Nunc, No. 163320 base), and polypropylene (PP) plates (Matrix Technologies, MT4918). The MPC plates were kindly provided from NOF corporation (Tsukuba, Japan). Flat-bottom 96-well polystyrene plates (Nunc, No. 167008) coated with MPC (f-MPC plate) were used for the comparison of the bottom shape of the culture plate. The PS plate is a tissue culture grade, in which cells are

easy to attach to the bottom of wells. Polypropylene of the PP plate is the material in which cells are relatively difficult to attach, compared with polystyrene.

ES cell culture

Mouse embryonic stem (ES) cells of the cell line 129SV were cultured on gelatin-coated dishes with feeder cells at 37 °C in humidified air with 5% CO₂. The immortalized STO fibroblasts were used as feeder layers (Shamblott et al. 2002). The STO cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% inactivated fetal bovine serum (FBS; Gibco), 25 units/ml penicillin, and 25 µg/ml streptomycin. The STO cells were treated with a freshly prepared solution of mitomycin C (Sigma, St. Louis, MO) at 10 µg/ml for 3 h to be incapable of division. Feeder layers for ES cells were prepared by replating the mitomycin C-treated STO cells at approximately 5×10^5 cells per 35 mm dish and incubated overnight. The ES cells (2×10^5 cells per 35 mm dish) were cultured on the feeder layers in DMEM (Gibco) supplemented with 15% KnockOutTM serum replacement (KSR; Gibco), 1 mM sodium pyruvate (Gibco), 0.1 mM nonessential amino acids (Gibco), 0.1 mM 2-mercaptoethanol (Sigma), 25 units/ml penicillin, and 25 µg/ml streptomycin (ES medium). The undifferentiated state was maintained by 1000 units/ml recombinant murine leukemia inhibitory factor (mLIF; Chemicon, Temecula, CA). For these experiments, ES cells were grown for a maximum of 22 passages in the presence of feeders and mLIF.

Embryoid body formation

For the EB formation cultures, ES cells, which were cultured for 3 days, were dissociated using 0.1% trypsin-EDTA (Gibco) and suspended in Iscove's modified Dulbecco's medium (IMDM; Gibco) supplemented with 15% inactivated FBS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 25 units/ml penicillin, and 25 µg/ml streptomycin (EB medium) at a cell density of 2×10^4 cells/ml. The mLIF was not added to the EB-forming cultures.

For the EB formation, the ES cells were seeded at various densities of 200, 1000, and 4000 cells per well in the MPC plates with 200 μ l EB medium. For the EB formation in the PS, the PP, and the f-MPC plates, the seeded cell density was at 1000 cells per well. The plates were incubated statically in the humidified CO₂-incubator (37 °C, 5% CO₂) for 5 days. The formed EBs were microscopically observed. The number of viable ES cells contained in an EB was counted with the hemocytometer after dissociating the EB by incubation in 0.25% trypsin-EDTA (Gibco). Cell viability was determined by the exclusion of Trypan blue dye.

Attached EB culture to generate cardiomyocytes

The EBs, which were formed from 200 ES cells (200-EB), 1000 ES cells (1000-EB), and 4000 ES cells (4000-EB) in the EB formation culture for 5 days, were transferred to the attachment culture for the differentiation of ES cells into cardiomyocytes. The EBs of the same culture age (5-days-old) were used in this experiment in order to make the differentiation time even. The attachment cultures were performed in the EB medium on a 24-well plate (IWAKI No. 3820-024N) coated with 0.1% gelatin. Twenty-four EBs were seeded on the 24-well plate to be one EB in one well. Periodically, microscopic observation was performed to detect the generation of the beating cells in the populations derived from an EB in each well. Three independent experiments were conducted on each EB type.

Immunohistochemical staining

To detect cardiac muscle (cardiomyocytes) in the outgrowth of EBs, immunohistochemical stain was performed. The cultured EBs were fixed with 10% formalin for overnight at 4 °C. The fixed sample was washed with phosphate buffered saline for 5 min at room temperature 3 times, and then incubated at 4 °C for overnight with the primary antibody (cardiac troponin I monoclonal antibody, QED Bioscience Inc., San Diego). The sample was incubated with secondary antibody (Simple Stain Mouse MAX-PO, Nichirei, Tokyo) for 30 min, and incubated with the color reagent

(Simple Stain DAB, Nichirei, Tokyo) for 5–20 min at room temperature. Finally, the sample was stained for 10 s with hematoxylin to reveal the nuclear of cells.

Statistics

At least three independent experiments were conducted. Values derived from replicate measurements were reported as the mean $\pm$ SD.

Results

EB formation in various culture plates

The EB formation was performed using various 96-well plates. In the MPC plate and the f-MPC plate, the formation of EB was observed without cell adhesion to the well. In the PS plate, the ES cells attached to the bottom of wells and no cell aggregation was observed (Figure 1a). In the PP plate, the ES cells slightly aggregated, but they did not form a spherical cell aggregate (Figure 1b). Figure 2 shows the typical EB forming process in a well of the MPC plate. ES cells formed a three-dimensional cell aggregate by day 1. The size of the cell aggregate gradually increased, and then it became an EB with about 550 μ m diameters by day 5. In most cases one EB was formed in a well of the MPC plate. There was the EB formation in all seeded 96 wells of the MPC plate. On the other hand, there was multiple EB formation in a well of the f-MPC plate and their sizes were heterogeneous.

When the EBs formed in the MPC plate were transferred to the attachment culture, the EBs were allowed to attach and spread out, and then the beating cells were observed in the outgrowth of the EBs. Figure 3 shows the result of the immunohistochemical staining of the outgrowth of the EB. The cytoplasm of the cells in the tissue around the beating area was stained in brown. The troponin-positive cells are ES cell-derived cardiomyocytes. As shown in Figure 3, the troponin-positive cells mainly appeared in the circumferential position of outgrowth from the center of EB. The troponin-positive area for the total area of EB outgrowth may be about 25% in a guess by the observation.

Figure 1. Photomicrographs of aggregates of ES cells in the PS plate and the PP plate after 5 days of EB formation culture. (a) PS plate, (b) PP plate. Scale bar is 200 μm .

Figure 2. Typical EB forming process in a well of the MPC plate. Scale bar is 200 μm .

Cell density for EB formation

The cultures for EB formation were performed at various seeding cell densities. Figure 4 shows the time course changes in the cell number contained in the EBs, which were 200-EB, 1000-EB, and 4000-EB. As increasing the cell density, the cell number reached the peak in the earlier time and the peak value increased. The peak values were approximately 13,000 cells in the 200-EB at day 5, 22,000 cells in the 1000-EB at day 5, and 26,000 cells in the 4000-EB at day 3. In any types of EBs, the cell number markedly decreased after day 5 of the culture.

Figure 5 shows the time course changes in the diameters of the EBs. The diameters of all of the EBs gradually increased with the elapse of the culture time. In the 4000-EB, the diameter reached the plateau, approx. 750 μm , by day 7.

The 200-EB and the 1000-EB were smaller than the 4000-EB, but the diameter of the 1000-EB approached the almost equal size to the 4000-EB by day 7. As shown in Figures 4 and 5, the decrease in the cell number of EBs after day 5 did not affect the diameters of EBs.

Cardiomyocytes differentiation from EBs

Differentiation efficiency of EBs to cardiomyocytes was investigated in the attached EB culture. Figure 6 shows the time courses of the percentage of wells where the beating was observed in the 24 wells attached EBs. In the attached culture of the 200-EBs, the beating was started in the relatively early time and the beating percentage reached 100% by day 7. As for the 4000-EBs, the beating start was delayed, and the beating

Figure 3. Immunohistochemical staining for troponin in the attached EB culture. Scale bar is 200 μm . The troponin-positive cells, of which cytoplasm was stained in brown, represents cardiomyocytes.

percentage was reduced. In the attached culture of the 1000-EBs, though the beating start was later than that of the 200-EBs, the time course of beating generation was most stable and beating percentage reached 100% by day 8.

Figure 4. Time course changes in the cell number contained in the EBs formed in the MPC plate. The EBs were initiated from 200 ES cells (200-EB), 1000 ES cells (1000-EB), and 4000 ES cells (4000-EB). Mean and standard deviation resulting from at least three experiments are shown.

Figure 7 shows the photographs of attached cultures of 200-ES, 1000-ES, and 4000-ES at day 7. The 200-EB rapidly spread out and completely lost three-dimensionality. The 1000-EB gradually spread out and flattened by day 7. In the attached culture of 4000-EB, cell spreading

Figure 5. Changes in the diameter of the EBs formed in the MPC plate. The EBs were initiated from 200 ES cells (200-EB), 1000 ES cells (1000-EB), and 4000 ES cells (4000-EB).

Figure 6. Time course changes in the percentage of wells where the beating was observed in the 24 wells. Mean and standard deviation resulting from at least three experiments are shown.

occurred in the outskirts of EB and three-dimensionality still remained on day 7.

Discussion

In the EB formation culture, the formation of cell aggregates is promoted by preventing cell attachment to tissue culture plastic. It is a point to notice that large cell aggregate in which necrosis happens is not formed. When standard suspension culture (ex. rotating suspension culture) is performed, cells and cell aggregates coalesce, ultimately forming very large and heterogeneous structures. There are some culture systems devised in order to form EBs of appropriate size. These include MC culture (Wiles 1993) and HD culture (Shamblott et al. 2002; Kurosawa et al. 2003b). Methylcellulose

medium (MCM) is semisolid medium. When ES cells are seeded into a semisolid matrix of MCM, they tend to remain stationary and develop EBs from single cells. However, it is not easy to handle semisolid MCM by pipettes, and in some cases, the MCM prevents the mass transfer. For the HD culture, 400–1000 cells are applied to the lid of culture dish as a droplet of 20–50 μ l medium volume. The droplets of ES cell suspension hang from the lid by the surface tension. Since medium volume of a droplet so small, it must be careful about the evaporation of the medium. The dish with the hanging droplets should be handled carefully so as not to disturb the hanging droplets. In the HD culture, therefore, it is difficult to observe the forming EBs in the middle point of the EB formation culture, and it is troublesome to pick up the formed EBs by pipettes.

The MPC coated surface, which is covered with the polymers containing phosphorylcholine polar groups, has the structure that resembles cell membrane (Yu et al. 1994). Therefore, the hydrophobic interaction between cell and culture surface is hardly generated, resulting in low cell adhesion. In the MPC plate (round-bottom), stable and efficient EB formation was performed. The ES cells spontaneously aggregated and then formed a single EB in a well of the MPC plate. There were heterogeneous and multiple formation of EB in the f-MPC plate (flat-bottom). It was supposed that the round-bottom was effective to gather the cells at one place in the MPC plate. There was no EB formation in the PS plate and the PP plate though they were the round-bottom plates. It was supposed that the cell non-adhesiveness of the PS plate and the PP plate was insufficient to form EB. Therefore, it seems that

Figure 7. Photomicrographs of attached cultures of 200-EB (a), 1000-EB (b), and 4000-EB (c) on day 7. Scale bar is 500 μ m.

the non-adhesiveness and the roundness of the well are important factors to form a good EB. The efficiency of EB formation in the MPC plate was quite stable and high, and the percentage of wells with forming EB in the wells seeded ES cells was almost 100%. It may be impossible to obtain such a high efficiency for EB formation in the HD method (Kurosawa et al. 2003b).

As for cell seeding density, unfavorable results were obtained on EB formation and cardiomyocytes differentiation when ES cell were seeded at 4000 cells per well. In the EB formation culture at high cell density, too large cell aggregate (or EB) was frequently formed. In the too large EB, necrosis may happen in the central region of EB due to the failure of mass transfer. That seems to be the reason why the decrease in the cell number happened in the EB formation culture without reducing the EB size (Figures 4 and 5) and the three-dimensionality of the 4000-EB remained in the attached EB culture (Figure 7). In this study, 5-days-old EBs were employed for the attached EB culture regardless of the cell seeding density in order to make the differentiation time even. Taking account for the cell number contained in each EB as shown in Figure 4, it may be appropriate for the 4000-EB that the EB formation culture is carried out for 3 days, because the cell number reached the peak by day 3 in the 4000-EB. Dang et al. (2002) has reported that the maximum cell number contained in a single EB was $28,000 \pm 9000$ cells per EB. The cell numbers in the 1000-EB and the 4000-EB were reaching the maximum level as mentioned by Dang et al. (2002). On the other hand, the cell number contained in the 200-EB was markedly few compared with the maximum level. However, cardiomyocytes were more efficiently generated from the 200-EB than the 1000-EB and the 4000-EB as shown in Figure 6. It should be noted that the cell number in the EB and the differentiation capacity of EB are unrelated.

Various types of cells except for cardiomyocytes could be seen in the outgrowth of the EBs. It was supposed that neural cells and endothelial cells were generated, but they were not identified in this study. To form three-dimensional structures of ES cells, EBs, is normally required in the initial step of *in vitro* differentiation of ES cells because a wide variety of cell types, including cardiomyocytes, can be differentiated from the EBs (Boheler et al. 2002). In this study we could observe the

generation of cardiomyocytes in two-dimensional differentiation culture of ES cells, without EB formation. But, the efficiency of cardiomyocyte generation was low and the reproducibility was not sufficient (data not shown).

In this article we presented the EB formation method using the MPC plate, which has the wells of round-bottom and of non-adhesive property. It was found that the MPC plate would be effective as an instrument for the EB formation.

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References

- Boheler K.R., Czyz J., Tweedie D., Yang H.-T., Anisimov S.V. and Wobus A.M. 2002. Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circ. Res.* 91: 189–201.
- Bradley A., Evans M., Kaufman M. and Robertson E. 1984. Formation of germ-line chimeras from embryo-derived teratocarcinoma cell lines. *Nature* 309: 255–256.
- Dang S.M., Kyba M., Perlingeiro R., Daley G.Q. and Zandstra P.W. 2002. Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. *Biotechnol. Bioeng.* 78: 442–453.
- Evans M. and Kaufman M. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292: 154–156.
- Ishihara K. and Iwasaki Y. 1998. Reduced protein adsorption on novel phospholipid polymers. *J. Biomat. Appl.* 13: 111–127.
- Keller G.M. 1995. *In vitro* differentiation of embryonic stem cells. *Curr. Opin. Cell Biol.* 7: 862–869.
- Koide N., Sakaguchi K., Koide Y., Asano K., Kawaguchi M., Matsushima H., Takenami T., Tanabe T., Shinji T., Mori M. and Tsuji T. 1990. Formation of multicellular spheroids composed of adult rat hepatocytes in dishes with positively charged surfaces and under other nonadherent environments. *Exp. Cell Res.* 186: 227–235.
- Kurosawa H., Yuminamochi E., Yasuda R. and Amano Y. 2003a. Morphology and albumin secretion of adult rat hepatocytes cultured on a hydrophobic porous expanded polytetrafluoroethylene membrane. *J. Biosci. Bioeng.* 95: 59–64.
- Kurosawa H., Imamura T., Koike M., Sasaki K. and Amano Y. 2003b. A simple method for forming embryoid body from mouse embryonic stem cells. *J. Biosci. Bioeng.* 96: 409–411.
- Martin G. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 78: 7634–7638.

- Shamblott M.J., Axelman J., Sternecker J., Christoforou N., Patterson E.S., Siddiqi M.A., Kahler H., Ifeanyi L.A. and Gearhart J.D. 2002. Stem cell culture: pluripotent stem cells. In: *Method Tissue Engineering*. Academic Press, San Diego, pp. 411–420.
- Ueda T., Watanabe A., Ishihara K. and Nakabayashi N. 1991. Protein adsorption on biomedical polymers with a phosphorylcholine moiety adsorbed with phospholipid. *J. Biomat. Sci. Polymer. Ed.* 3: 185–194.
- Watanabe J. and Ishihara K. 2003. Phosphorylcholine and poly(D,L-lactic acid) containing copolymers as substrates for cell adhesion. *Artif. Organs* 27: 242–248.
- Wiles M.V. 1993. Embryonic stem cell differentiation in vitro. *Methods Enzymol.* 225: 900–918.
- Yu J., Lamba N.M., Courtney J.M., Whateley T.L., Graylor J.D., Lowe G.D., Ishihara K. and Nakabayashi N. 1994. Polymeric biomaterials: influence of phosphorylcholine polar groups on protein adsorption and complement activation. *Int. J. Artif. Organs* 17: 499–504.
- Zhu A., Wang S., Yuan Y. and Shen J. 2002. Cell adhesion behavior of chitosan surface modified by bonding 2-methacryloyloxyethyl phosphorylcholine. *J. Biomat. Sci. Polymer. Ed.* 13: 501–510.