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A feeder-free, defined three-dimensional polyethylene glycol-based extracellular matrix niche for culture of human embryonic stem cells

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ABSTRACT

We report optimization of a serum- and feeder-free, three-dimensional (3D) niche created with a synthetic polyethylene glycol (PEG)-based extracellular matrix for self-renewal of human embryonic stem cells (hESCs). Three hESC lines (H9, H1 and Novo) were cultured in hydrogels of different mechanical properties, and cellular morphology and activity were compared to culture in feeder-free or feeder-containing two-dimensional (2D) niches. Significant effects of PEG concentration (5, 7.5, 10, 12.5 or 15%) and vinyl sulfone-functionalized PEG multiarm number (3, 4 or 8) on hESC morphology were detected in the H9 line. Cell growth was maximal with an 8 multiarm architecture of any PEG concentration, which yielded the highest expression of stemness-related genes. Alkaline phosphatase activity in cultured H9 cells was similar between the optimized feeder-free 3D and the feeder-containing 2D systems. However, increased expression of the *KLF4*, *CDH1*, *TERT*, *SOX2*, and *UTF1* genes and expression of pluripotency-specific SSEA-4, Oct3/4, Nanog, Tra-1-60 and Tra-1-81 were detected in the 3D-cultured hESC clumps. H1 and Novo cell lines also expanded in the optimized 3D system, which maintain stemness properties. Although different proliferation activities were detected among three lines, the difference was decreased after the 3D culture. These results demonstrate that chemically defined, acellular niches created using PEG-based hydrogels have the potential to support hESC self-renewal. Modulation of 3D properties can create various models for cell transformation and differentiation.

1. Introduction

Development of biomaterial matrices is important in the field of stem biotechnology for both preclinical and clinical applications, including model development, mechanism discovery and clinical engineering. Conventional two-dimensional (2D) systems for stem cell culture provide a suboptimal extracellular matrix (ECM) environment and non-physiological cell signaling, thus limiting the ability to induce specific signals for self-renewal or differentiation pathways. Recently, the importance of cellular and acellular niches that specifically regulates cell fate and cellular function has become emphasized [1]. The stem cell niche creates a unique microenvironment for regulating pluripotency and stem cell self-renewal. However, the precise biomolecular composition and function of the niche have not been clarified. We previously suggested the use of feeder-free, defined three-dimensional (3D) systems for the embryonic stem cell (ESC) culture with murine cells, utilizing hydrogels formed by crosslinking reactive polyethylene glycol (PEG)-based macromers [2]. The physical properties of this synthetic ECM could be easily modified by altering the PEG concentration and number of arms on the PEG-vinyl sulfone (VS) macromer. Various peptide sequences can be conjugated to the PEG-based, artificial ECM to provide either activation or inhibition signals. Thus, the matrix biomaterial may be of broad use in stem cell culture and processing.

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In this study, we explore the utility of the PEG-based matrix in culture of human ESCs (hESCs). We employed multiple lines of hESCs to evaluate feasibility of feeder-free self-renewal in the PEG-based hydrogel creating a 3D environment. Cellular morphology and activity of the hESCs cultured in the biomechanically optimized 3D system were compared with those obtained by cultured in feeder-free or feeder-containing 2D systems.

2. Materials and methods

2.1. Experimental design

hESCs of H1, H9 and Novo lines were obtained from a commercial source (WiCell Research Institute; Wilmington, MA). Since the hydrogel matrix is sensitive to matrix metalloproteinases (MMPs), expression of *MMP* isotypes by hESCs was preliminary determined.

In a first series of experiments, the elastic modulus of the matrix was optimized, which was accomplished by using hydrogels formed from different VS-functionalized multiarm PEG macromers (3, 4 or 8 arms per branched chain) and/or PEG concentrations (7.5, 10, 12.5 or 15%). H9 hESCs encapsulated within the hydrogels were freed as described below from the 3D system on day 9 of encapsulation to count the enlarged clumps. To characterize cellular and physiological activity after the 3D culture, the activity of alkaline phosphatase (AP), protein expression of stage-specific embryonic antigen (SSEA)-1, SSEA-4, Oct3/4, Nanog, Tra-1-60 and Tra-1-81, and mRNA expression of the stemness-related genes *OCT3/4*, *NANOG*, *KLF4*, *CDH1*, *TERT*, *SOX2*, and *UTF1* were evaluated in H9 hESCs cultured in 3D or 2D systems in the second series of experiments. Cultured cells were also scanned with a confocal microscope to evaluate maintenance of an undifferentiated state in a whole matrix of hESC clumps. H9 hESCs maintained under the optimal condition were karyotyped on culture day 9. Differentiation was induced in H9 hESCs and the expression levels of mesodermal (smooth muscle actin; SMA and Desmin), endodermal (α-fetoprotein; AFP and Troma-1), and ectodermal (N-cadherin and Nestin) cell markers were monitored. *In vivo* differentiation was estimated based on teratocarcinoma formation. In the last series of experiments, H1 and Novo hESCs were also cultured in the 3D system to confirm the utility of the 3D culture system obtained by optimization using the H9 cell line. Cell proliferation and morphology during 3D culture were compared.

2.2. Guideline and preparation for experimentation

All procedures were conducted according to the standard operating protocols of the human stem cell laboratory at Seoul National University and the ethical guidelines for research and experimentation. The facility for manipulating hESCs was designed by the Ministry of Health and Welfare, Republic of Korea. IRB review was exempted because of utilization of hESCs from a commercial source according to the guideline of Seoul National University IRB committee. The quality control of experimental procedures and data management was conducted by our standard operation protocol.

2.3. Culture of hESCs

The hESCs were initially cultured on mouse embryonic fibroblast (MEF) feeder layers. The cells were mechanically transferred every 4–5 days. Undifferentiated hESCs were cut away from the feeders as small clumps. Undifferentiated hESCs, identified by their morphology, were chosen for each further passage. In the 2D feeder-free system, hESCs were cultured in culture dishes coated with Matrigel (BD Biosciences, San Diego, CA). The culture medium consisted of 80% (*v*/*v*) DMEM/F12, 20% (*v*/*v*) knockout serum replacement (KSR), 10 mM nonessential amino acids (all from Gibco-Invitrogen, Cergy-Pontoise, France), 50 μM β-mercaptoethanol (Sigma–Aldrich, St. Louis, MO), and 4 ng/ml basic FGF (Gibco-Invitrogen). Cells were incubated at 37 °C and 95% humidity in a 5% CO_2/95% air environment. The culture medium was changed daily.

2.4. Formation of PEG-based hydrogels and insertion of hESCs

PEG-based hydrogels were formed as described previously [2]. In this study, we used 3-, 4-, and 8-arm PEG-VS, and undifferentiated hESC clumps that collected in the hESC culture medium were encapsulated. The medium was exchanged every other day. To release the encapsulated hESCs, PEG hydrogels were incubated for 20 min in hESC culture medium containing 10 μg/ml collagenase type I (Sigma–Aldrich).

2.5. Analysis of relative mRNA levels by real-time PCR

hESCs were transferred to RNAlater® (Ambion, Austin, TX) and stored at −75 °C until required. Total mRNA was subsequently extracted using an RNeasy® Mini Kit (Qiagen, Valencia, CA). The cDNA was synthesized from total RNA using SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The expression of specific genes was quantified by real-time PCR using a CFX96 Real-Time Detection System (Bio-Rad, Hercules, CA) using a DyNAmo HS SYBR Green qPCR Kit (Finnzymes Oy, Espoo, Finland). PCR amplification was performed through 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Melting curve analysis was performed to check PCR specificity. β-actin expression was measured in each treatment group for standardization, and the expression level of each target mRNA was normalized to that of the β-actin mRNA. Relative mRNA level was calculated as $2^{-\Delta Ct}$, where Ct = the threshold cycle for target amplification and $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{internal reference}}$ (β-actin).

2.6. AP activity

AP activity was measured using an ES Cell Characterization Kit (Chemicon, Billerica, MA) according to the manufacturer's protocol. Stained cells were imaged with an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan) equipped with a Jena ProgRes® camera (Jenoptik, Jena, Germany).

2.7. Immunostaining

To detect the pluripotency-related surface markers SSEA-1, SSEA-4, Tra-1-60, and Tra-1-81 (all from Chemicon), hESCs were fixed with 4% paraformaldehyde (Sigma–Aldrich) in phosphate-buffered saline (Welgene, Daegu, Korea) and stained according to the manufacturer's protocol. To detect the pluripotency-related transcription factors Oct3/4 (Santa Cruz Biotechnology, Santa Cruz, CA) and Nanog (Chemicon), fixed cells were permeabilized with 0.1% Triton X-100. Fluorescence was visualized using an Eclipse TE2000-U microscope (Nikon) equipped with a Jena ProgRes® camera (Jenoptik). Images of stained cells were analyzed using an FV-300 confocal scanning system (Olympus, Tokyo, Japan).

2.8. Karyotyping

To analyze hESC karyotypes on day 9, cell division was blocked at metaphase through treatment with 0.1 g/ml colcemid (Gibco-Invitrogen) for 4 h. Cells were then trypsinized, resuspended in 0.075 M KCl, incubated for 20 min at 37 °C, and fixed in a 3:1 methanol:acetic acid mixture. G-band standard staining was used to visualize the chromosomes. Karyotypes were analyzed using a Karyo Chromosome Image Processing System (GenDix, Inc. Seoul, Korea).

2.9. In vitro differentiation of hESCs

hESCs cultured in PEG hydrogels were collected on day 9 and cultured for 2 weeks in hESC culture medium (without bFGF) in gelatin-coated tissue culture dishes. Differentiated hESCs were stained for the following germ layer markers: SMA (Biodesign International, Saco, ME), Desmin (Santa Cruz Biotechnology), AFP (Santa Cruz Biotechnology), Troma-1 (Hybridoma Bank, Iowa City, IA), N-cadherin (Santa Cruz Biotechnology), and Nestin (Chemicon). Bound antibody was detected using a kit from DakoCytomation (Carpinteria, CA).

2.10. In vivo differentiation of hESCs

hESC clumps cultured in PEG hydrogels were collected and subcutaneously co-injected with Matrigel (BD Biosciences) into 8-week-old severe combined immunodeficiency (SCID) mice. Nine weeks post-injection, the SCID mice were killed and the cell masses dissected, fixed in 4% formalin, and stained with hematoxylin and eosin. The stained tissues were examined histologically.

2.11. Cell proliferation assay

Proliferation of hESCs was monitored with a colorimetric cell counting kit (CCK-8; Dojindo molecular technologies, Gaithersburg, MD). hESCs were seeded in the feeder-containing 2D and the feeder-free 3D systems being optimized for the culture of the H9 hESC line. Two days after culture, the hESCs of different cell lines were treated with 10% (*v*/*v*) CCK-8 reagent and the color reaction 2 h after treatment was determined at the absorbance of 490 nm using a microplate reader (Model-550; Bio-Rad, Bio-Rad Lab. Inc., Hercules, CA).

2.12. Statistical analysis

All numerical data obtained in each experiment were analyzed statistically using Statistical Analysis System software (SAS Institute, Cary, NC). When a significant main effect was detected by analysis of variance (ANOVA) using SAS, the least-squares and Duncan methods were used to compare the different treatments. Differences between treatments were deemed to be significant when $p < 0.05$.