Investigations of Chirality Effects on Undifferentiated State of Mesenchymal Stem Cells Using Soft Nanofibrous Oligopeptide Hydrogels

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Soft nanofibrous oligopeptide hydrogels consisting of self-assembled L- and D-form Fmoc-Phe-Phe-Cys networks photo-cross-linked by poly(ethylene glycol)-dimethacrylate, which are referred to as L- and D-gel, respectively, were developed for investigation of chirality effects on the undifferentiated state of mesenchymal stem cells. Encapsulated mesenchymal stem cells in D-gel showed slower growth and less spreading, resulting in higher maintenance of the undifferentiated state, compared to in L-gel. This indicates that D-form peptide materials might be useful as scaffold materials for regenerative medicine using stem cells.

Keywords Chirality, 3D culture, mesenchymal stem cell, peptide self-assembly, fibrous hydrogels, cell spreading, undifferentiated state

Introduction

Mesenchymal stem cells (MSCs) have attracted much attention as a promising source for cell-based therapy and regenerative medicine since MSCs are able to self-renew and differentiate into a variety of cells, such as osteocytes, adipocytes, and nerve cells due to microenvironment around the cells and various stimulations.14 Transplantation of MSCs has been widely studied for treatments of neurological, cardiovascular, and immunological diseases. Recently, intravenous infusion of MSCs has been used in clinical trials for treatments of devastating neurodegenerative diseases6 and coronavirus disease 2019 (COVID-19).7 One of therapeutic abilities of MSCs is due to their potential to differentiate into desired cell types at the injured areas. On the other hand, paracrine factors secreted by undifferentiated MSCs play important roles in the therapeutic benefits of these cells by protecting damaged cells and increasing angiogenesis.8 However, MSCs lose their undifferentiated state as the result of long-term cell culture.9 Therefore, maintenance of the undifferentiated state in MSCs is one of the important issues for achieving maximum effectiveness in stem cell therapy. To expand the MSCs with multipotency, the development of new scaffold materials has been desired for the in vitro expansion of MSCs and the further enhancement of MSC-based therapeutic efficiency.

Hydrogels are attractive as three-dimensional (3D) scaffolds for not only expansion of a therapeutically relevant number of stem cells but also cell transplantation.10 Although numerous hydrogel scaffolds have so far been reported,11 self-assembling nanofibrous oligopeptide hydrogels are promising materials due to ordered superstructures, good biocompatibility, and biofunctionality.12 However, the self-assembling structure of oligopeptide is unstable under the physiological condition, making them unsuitable for long-term cell culture.13 To overcome this issue, recently, we developed a hydrogel culture system constructed by self-assembled L- and D-form oligopeptide nanofiber, Fmoc-Phe-Phe-Cys (L-Fmoc-FFC), crosslinked by poly(ethylene glycol)-dimethacrylate (PEG-DMA), which are referred to as L- and D-gel, respectively (Fig. 1(a)).13 It was confirmed that the physical properties of both gels were exactly the same except for the chirality, and their stiffness was confirmed that the physical properties of both gels were exactly the same except for the chirality, and their stiffness was approximately 200 Pa, which is classified as a soft hydrogel.13 Crosslinkage between oligopeptide fibers with PEG-DMA was carried out by ultraviolet (UV) irradiation via thiol–ene reaction. As shown in Fig. 1(b), the hydrogels without UV exposure disappeared immediately after the addition of hydrogels into the culture medium. While the hydrogels with UV exposure did not collapse even when shaking them into the culture medium. Utilizing the photo-crosslinking of PEG chains between self-assembling oligopeptides, soft fibrous hydrogels permit long-term cell culture. In a previous study, we investigated the chirality effects on osteo- and adipo-differentiation of MSCs using both L- and D-gel.13 Interestingly, the behavior of the encapsulated MSCs was drastically changed by the chirality of amino acids in oligopeptide nanofibers: the cells in D-gel showed

Notes

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slower growth and less spreading, resulting in lower ability of osteo- and adip-differentiation under mixed induction medium of osteogenesis and adipogenesis. Recently, it has been reported that the undifferentiation of MSCs is affected by cell spreading. MSCs gradually lose their undifferentiated state when MSCs are in an environment where they can spread. Therefore, to culture MSCs with undifferentiated state, scaffold materials that can suppress the cell spreading are required. Here, we hypothesized that the cell culture in d-gel might be suitable for maintaining the undifferentiated state of MSCs. In this study, we investigated the effect of chirality in the oligopeptide nanofiber on the maintenance of the undifferentiated state.

Experimental

Cell encapsulation in the hydrogels

The peptides were synthesized by solid-phase peptide synthesis using 2-chlorotrityl chloride resin, according to the procedure described in a previous report. After l-Fmoc-FFC or d-Fmoc-FFC was dissolved in PBS by adjusting the pH value, PEG-DMA and photoinitiator, Irgacure D-2959, were added to the oligopeptide solution. A cell line of human bone marrow-derived mesenchymal stem cells, UE7T-13 cells (JCRB Cell Bank, Osaka, Japan), suspended in PBS was added to the mixture of Fmoc-FFC, PEG-DMA, and Irgacure D-2959, followed by gentle shaking. The concentrations of oligopeptide, PEG-DMA, and Irgacure D-2959 were 0.5, 3.5, and 0.05 wt%, respectively. The cell seeding density was approximately 1.5 × 10^6 cells/mL. Forty-five microliters of the solution were pipetted into the top of a 1-mL syringe mold, followed by UV irradiation (365 nm, 5 mW/cm²) for 5 min. Then, hydrogels were washed with DMEM, and cultured in growth medium, which contained 10% fetal bovine serum (FBS, S1820-500, Biowest, France) and 1% antibiotic penicillin in DMEM (high glucose, with L-Glutamine and Phenol Red, 044-29765, Wako, Japan). The CO₂ concentration and temperature in the incubator were kept at 0.5% and 37°C.

MTT assay for measurement of cell proliferation

The tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution prepared in PBS was added to the medium and the cell-gel constructs were further incubated at 37°C for 4 h. Final concentration of MTT was 1%. The dark blue crystals of formazan were extracted with 0.04 N HCl in isopropanol at 25°C for 1 h. Quantification of formazan was performed using a UV/vis spectrometer (Ultroscopy 3300 pro, Amersham Biosciences, Amersham, UK) at 560 nm.

Assessment of mRNA expression

The gels were first mechanically homogenized in a 1.5-mL microcentrifuge tube containing RLT buffer and further homogenized with a QIA shredder (Qiagen, Hilden, Germany)
column. Then RNA was isolated according to the manufacturer’s protocol using the RNasey mini kit. cDNA was generated in a reverse transcription reaction using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) and random primers. Real-time PCR was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo) with StepOnePlus™ (Thermo Fisher Scientific, Waltham, MA). The relative mRNA levels were calculated by the ΔΔCt method and normalized to GAPDH. The primer sets are shown in Table S1 (Supporting Information).

Culture of MSCs on the gel surfaces

The glass surface was immersed in 7.5 wt% KOH/MeOH, followed by sonication for 20 min. After that, the glass surface was immersed in distilled water for 10 min with sonication. The glass surface was modified with an ethanol solution of 3-(trimethoxysilyl)propyl methacrylate (2 vol%) for 3 min and gently rinsed with methanol. The vinyl group-introduced glass surface thus prepared was modified with an ethanol solution of 3-aminopropyltrimethoxysilane (1 vol%) for 3 min. Then, the glass was washed with deionized water three times and allowed to stand for 60 min at 120°C. The mixture including 0.25% oligopeptide nanofiber, PEG-DMA (MW: 4600, 25 mg) and Irgacure 2959 (2.5 mg) in PBS was dropped on the silanized glass surface, followed by UV irradiation for 10 min (254 nm, 2400 mJ/cm²). The glass was washed with deionized water three times. To culture the cells on the gel surface, the cells (5 × 10⁵ cells/mL) were added on gel-coated glass in the culture dishes. After incubation for 48 h, cell attachment and morphology were observed with a phase-contrast microscope, using a Keyence BZ-X710 microscope (Osaka, Japan) with a 20× objective lens.

Results and Discussion

The MSCs were encapsulated in the l- and d-gel and cultured in growth medium to investigate the maintenance of the undifferentiated state. A previous report showed that, after four weeks of culture in growth medium, nearly all cells were alive in both l- and d-gel, as determined by live/dead staining. The proliferation of cells encapsulated in the hydrogels was examined by MTT assay. After two weeks of culture in growth medium, the rates of cell growth in l- and d-gel were nearly the same (Fig. 2(a)). In contrast, after four weeks of culture in growth medium, the proliferation rate of the cells in l-gel was greater than that in d-gel, and the cell growth in l-gel was approximately 2-fold more than that in d-gel (Fig. 2(a)). Because hydrogel culture environment factors, including chemical composition, structure, and stiffness, were nearly the same between l- and d-gel, the difference in cell proliferation can be attributed to the different chirality of the amino acids in oligopeptide nanofibers.

To investigate the influences of the chirality of amino acids in the nanofibrous oligopeptide hydrogels on the undifferentiated state of MSCs, measurement were taken of the mRNA levels of MSC biomarkers (b) ALCAM, (c) CD63, and (d) CD271 of MSCs in l- and d-gels 2 and 4 weeks after being cultured in growth medium. The relative mRNA levels are expressed as the value relative to that just after the encapsulation at day 0. *P < 0.05. n = 3.
After 2 weeks of culture, the mRNA levels of ALCAM, CD63 and CD271 were similar in l- and d-gel. However, after 4 weeks, the relative mRNA levels of ALCAM, CD63 and CD271 in l-gel significantly decreased to 0.59, 0.27, and 0.47, while those in d-gel slightly decreased to 0.90, 0.83, and 0.85. This result indicates that UE7T-13 cells diminished the undifferentiated state during long-term culture in l-gel.

Since the microscopic observation of cell morphology encapsulated in the hydrogel was unclear,15 the cell culture on the gel surfaces was conducted (Fig. 3). Interestingly, after 48 h of culture on the hydrogel surface in growth medium, the cells spread more on the l-gel surface than that on the d-gel surface. This result clearly showed the difference of cell spreading due to different chirality of amino acids in the oligopeptide nanofibers. In contrast, on PEG gel with non-adhesive property, the cells possessed a rounded morphology (Fig. 3). By comparing cell behavior on d-gel and PEG gel, it was apparent that d-gel surfaces were more adhesive, compared with PEG gel surfaces. From these results, we concluded that d-gel must be an adhesive surface where cell spreading is unlikely to occur.

Thus far, using the two-dimensional culture systems, the characteristics of MSCs, which remain undifferentiated, have been reported.15,16 On the cell adhesive surfaces, such as glass, MSCs enhance cell spreading and exhibit high cytoskeletal tension, leading to the increased proliferation and loss of the undifferentiated state. This is similar to the phenomenon of the cells in l-gel. In contrast, on the cell adhesive surfaces that suppress cell spreading, the cells with lower cytoskeletal tension grow slowly and maintain the undifferentiated state.14,16 In the present study, we found that the cell encapsulation in d-gel is able to accomplish slower cell proliferation and higher maintenance of the undifferentiated state for a month because of less cell spreading. Since d-gel consisted of unnatural amino acids, scaffold-cell interaction might be weak on d-formed oligopeptide nanofiber, resulting in less cell spreading. In conclusion, we found that the cell culture in d-gel strongly maintained the undifferentiated state for a month. Since expansion of the MSCs with the undifferentiated state is important for the success of cell-based therapy, the material using d-form peptide might be useful as a scaffold for regenerative medicine using stem cells.

Acknowledgements
This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science KAKENHI [grant numbers: 16K16398 and 19H05458 (to T. Y.).]

References
1. J. Yoshioka, T. Yoshitomi, T. Yasukawa, and K. Yoshimoto, Anal. Sci., 2016, 32, 1213.
2. Y. Furuhata, T. Yoshitomi, Y. Kikuchi, M. Sakao, and K. Yoshimoto, ACS Appl. Mater. Interfaces, 2017, 9, 9339.
3. X. Jia, K. Minami, K. Uto, A. C. Chang, J. P. Hill, J. Nakanishi, and K. Ariga, Adv. Mater., 2020, 32, e1905942.
4. X. F. Jia, K. Minami, K. Uto, A. C. Chang, J. P. Hill, T. Ueki, J. Nakanishi, and K. Ariga, Small, 2019, 15, e1804640.
5. Y. W. Eom, J. E. Oh, J. I. Lee, S. K. Baik, K. J. Rhee, H. C. Shin, Y. M. Kim, C. M. Ahn, J. H. Kong, H. S. Kim, and K. Y. Shim, Biochem. Biophys. Res. Commun., 2014, 445, 16.
6. N. P. Staff, D. T. Jones, and W. Singer, Mayo Clin. Proc., 2019, 94, 892.
7. F. Meng, R. Xu, S. Wang, Z. Xu, C. Zhang, Y. Li, T. Yang, L. Shi, J. Fu, T. Jiang, L. Huang, P. Zhao, X. Yuan, X. Fan, J. Y. Zhang, J. Song, D. Zhang, Y. Jiao, L. Liu, C. Zhou, M. Mauer, A. Zuml, M. Shi, and F. S. Wang, Signal. Transduct. Target Ther., 2020, 5, 172.
8. Y. L. Tang, Q. Zhao, X. Qin, L. Shen, L. Cheng, J. Ge, and M. I. Phillips, Ann. Thorac. Surg., 2005, 80, 229.
9. D. S. Yoon, Y. H. Kim, H. S. Jung, S. Paik, and J. W. Lee, Cell Proliferation, 2011, 44, 428.
10. A. M. Akimoto, E. Hasuike, H. Tada, K. Nagase, T. Okano, H. Kanazawa, and R. Yoshida, Anal. Sci., 2016, 32, 1203.
11. S. Naahidi, M. Jafari, M. Logan, Y. Wang, Y. Yuan, H. Bae, B. Dixon, and P. Chen, Biotechnol. Adv., 2017, 35, 530.
12. Z. W. Shen, Z. Guo, T. Y. Tan, J. Hu, and Y. Zhang, ACS Biomater. Sci. Eng., 2020, 6, 3957.
13. H. Zheng, T. Yoshitomi, and K. Yoshimoto, ACS Appl. Bio Mater., 2018, 1, 538.
14. R. J. McMurray, N. Gadegaard, P. M. Tsimbouri, K. V. Burgess, L. E. McNamara, R. Tare, K. Murawski, E. Kingham, R. O. Oreffo, and M. J. Dalby, Nat. Mater., 2011, 10, 637.

15. J. W. Song, X. F. Jia, K. Minami, J. P. Hill, J. Nakanishi, L. K. Shrestha, and K. Ariga, ACS Appl. Nano Mater., 2020, 3, 6497.

16. X. Wang, T. Nakamoto, I. Dulinska-Molak, N. Kawazoe, and G. Chen, J. Mater. Chem. B, 2016, 4, 37.

17. M. Alvarez-Viejo, Y. Menendez-Menendez, and J. Otero-Hernandez, World J. Stem Cells, 2015, 7, 470.