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論　文　内　容　の　要　旨

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論文題名	Virus-Free and Autologous Culture System for Human Gingiva-Derived iPS Cells (歯肉を用いた動物由来成分／ウイルス・フリーのヒトiPS細胞培養技術の確立)

論文内容の要旨

Introduction

Induced pluripotent stem cells (iPSCs) are expected to be a powerful tool for regenerative medicine; however, the clinical applicability of these cells still has to be carefully investigated. A potential drawback of iPSCs used clinically is the possibility of tumor formation after *in vivo* implantation arising from unexpected insertional mutagenesis by viral vectors during iPSC generation. In addition, exposure of iPSCs to animal-derived materials, such as mouse feeder cells and bovine serum-containing media, may pose a risk for contamination by animal-derived pathogens. Many studies to generate and maintain iPSCs without using viral vectors and animal products have been undertaken; however, an optimal virus- and xeno-free culture method to safely generate iPSCs has not yet been determined.

Human gingival fibroblasts (hGFs) represent an ideal autologous cell source that can be easily collected by dentists, grown in a simple culture system, and easily reprogrammed into iPSCs. In addition, hGFs possess high proliferation ability and contribute to favorable early wound closure by expressing distinctive genes.We hypothesized that these unique characteristics of hGFs would favorably affect their reprogramming efficiency when using viral-free methods and also provide an advantage in the establishment of an autologous culture system in which hGFs are used as an alternative to the mouse-derived feeder cells.To test this hypothesis, we investigated whether hGFs can be used as autologous feeder cells for iPSC generation and culture without viral or animal-derived components. The aim of this study was to establish a virus- and xeno-free culture system for human iPSCs using hGFs as an iPSC source and as autologous feeder cells.

Material and method

1. Cell culture of hGFs in serum-free medium

We first investigated whether primary hGFs could be isolated under serum-free conditions. To establish hGF cultures, gingival tissues from patients were placed on a cell culture dish and maintained in serum-free growth medium (HFDM-1 medium). We compared the proliferation of hGFs and human dermal fibroblasts in growth medium consisting of DMEM supplemented with 10% fetal bovine serum (FBS growth medium) of serum-free growth medium using the WST-1 cell proliferation assay.

2. Application of hGFs to feeder cells for iPSC culture

To evaluate the ability of hGFs to sustain the undifferentiated status of iPSCs, hGF-derived iPSCs (hGF-iPSCs) were seeded on mitomycin-C-inactivated hGFs, hDFs, or mouse SNL feeder cells (SNLs). After 14 days, the undifferentiated status of the iPSCs was evaluated by alkaline phosphatase (ALP) staining, and the total area of the intact undifferentiated colonies was analyzed using ImageJ software. To investigate the long-term maintenance of undifferentiated iPSCs by hGF feeder cells, hGF-iPSCs were cultured on mitomycin-C-inactivated hGFs or SNLs and repeatedly passaged for 6 months. The undifferentiated status of hGF-iPSCs at 20 passages was confirmed by RT-PCR, bisulfite genomic sequencing, immunocytochemistry, and teratoma assay.

3. Generation of iPSCs using episomal plasmid vectors

For nonviral iPSC generation, hGFs were transfected with episomal plasmid vectors encoding (1) OCT3/4 and anti-p53 shRNA, (2) SOX2 and KLF4, and (3) L-MYC and LIN28 by electroporation, and cultured on mitomycin-C-inactivated hGFs, SNLs, or feeder-free culture plates coated with recombinant E8 fragments

of laminin isoforms (LM-E8). Upon emergence, embryonic stem cell (ESC)-like colonies were transferred onto hGFs or SNL feeder cells, or onto LM-E8-coated plates, to establish clonal iPSC cultures. To investigate the reprogramming efficiency of the nonviral method, hGFs and hDFs were transfected with the episomal plasmid vectors and transferred onto SNLs. The reprogramming efficiency of hGFs and hDFs was analyzed by counting ESC-like colonies positively stained with ALP.

4. Expression of insulin-like growth factor (IGF)-Ⅱ and laminin α5 in hGFs

IGF-Ⅱ and laminin α5 have been reported to contribute to self-renewal and maintenance of the undifferentiated state in ESCs. Expression of laminin α5 in hGFs and hDFs was examined by immunocytochemistry. Expression of IGF-Ⅱ from hGFs and hDFs was measured by Western blotting.

Results

1. Cell culture of hGFs in serum-free medium

In the serum-free growth medium, fibroblasts and epithelial cells migrated out of the gingival tissues. After several passages, epithelial cells were detached and spontaneously eliminated from the culture, and only GFs were isolated. The WST-1 assay showed that the amount of hGFs on day 7 was significantly higher than that of hDFs (ANOVA; $P<0.01$) in both FBS growth medium and serum-free growth medium.

2. Application of hGFs to feeder cells for iPSC culture

The area covered by undifferentiated hGF-iPSC colonies on hGF feeder cells on day 14 was significantly higher than that on SNLs (ANOVA; $P<0.01$). Most hGF-iPSCs on hDF feeder cells differentiated within 7 days. In long-term cultures, the feeder layers of hGFs and SNLs maintained the undifferentiated state of hGF-iPSCs for more than 50 passages. hDFs did not support the long-term undifferentiated state of hGF-iPSCs. ESC marker expression and CpG methylation at the Nanog and OCT3/4 promoters after 10 passages were similar for hGF-iPS cells cultured on hGFs and SNLs. hGF-iPSCs cultured on hGF feeder cells demonstrated *in vitro* and *in vivo* differentiation into various types of cells and tissues from the three primary germ layers.

3. Generation of iPSCs using episomal plasmid vectors

ESC-like colonies emerged within 21 days after plasmid transfection on hGF and hDF feeder cells or LM-E8-coated plates. The reprogramming efficiency of cells cultured on hGFs was significantly higher than that of cells cultured on hDFs (ANOVA; $P<0.01$). After colony pickup, hGF-iPSC colonies on hGF and SNL feeder cells were able to be further passaged and expanded to establish iPSC clones; however, hGF-iPSC colonies on LM-E8-coated plates differentiated within two passages and clonal cultures could not be established.

4. Expression of IGF-Ⅱ and laminin α5 in hGFs

Immunocytochemistry for laminin α5 indicated higher staining intensity in hGFs than in hDFs. The rate of positive staining for laminin α5 was higher in hGFs than in hDFs (ANOVA; $P<0.01$). Western blotting demonstrated that IGF-Ⅱ expression in hGFs was significantly higher than that in hDFs (ANOVA; $P<0.01$).

Conclusions

These results suggest that hGFs have superior proliferation ability that permits facile collection of a large number of cells using serum-free medium. We found that hGFs permit long-term maintenance of undifferentiated iPSCs, possibly through high expression of laminin α5 and IGF-Ⅱ. In addition, autologous hGF feeder cells are appropriate for non-viral iPSC generation, especially in the iPSC cloning step to amplify a small number of iPSCs while sustaining their undifferentiated state. The effective virus-free and autologous culture of hGF-iPSCs presented here thus represents an important step toward the future therapeutic usage of hGF-iPSCs.

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論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

本研究において、論文審査申請者（于　冠男）は、ヒト歯肉線維芽細胞は動物由来成分を含まない培地を用いても培養が可能であることを示した。また、患者由来の歯肉線維芽細胞にエピソーマルベクターを遺伝子導入することにより、ウイルスベクターを用いないで iPS 細胞の樹立が可能であることを明らかにした。さらに、ヒト歯肉線維芽細胞が iPS 細胞の自己フィーダーとして適した細胞であることを明らかにし、この分子機構には laminin α5 および IGF-II が関与している可能性を示唆した。以上の知見は、ヒト歯肉組織からウイルスおよび動物由来成分を用いないで iPS 細胞を作製し、培養を維持する一連のプロトコルを示している。本研究によって得られた医療応用に安全な歯肉由来 iPS 細胞技術は、将来的に再生歯科医療に貢献することが期待される。よって、博士（歯学）の学位論文として価値のあるものと認める。