早稲田大学大学院先進理工学研究科

博 士 論 文 概 要

論 文 題 目

Elucidation and Application of Effective Factors of Epithelial Cell Culture for Regenerative Medicine

再生医療本格化のための上皮細胞培養系における有効因子の解明とその応用

申 請 者

Makoto	KONDO
近藤	誠

生命理工学専攻　生体制御研究

2012 年 12 月

There has been a large upsurge in epithelial regenerative medicine in recent 30 years, giving a novel approach for overcoming the issues of symptomatic therapies and surgical treatments. Temperature responsive cell culture surfaces allow confluently cultured cells to detach themselves from the surfaces as a contiguous cell sheet non-invasively. Oral mucosal epithelial cells have been employed to treat corneal and esophageal regeneration, because these cells are classified in stratified squamous epithelial cells. Conventional culture method for stratified squamous epithelium has used xenogeneic material, such as 3T3 feeder cells, to enhance their cell growth. By using temperature responsive cell culture insert, the fabrication of dog oral mucosal epithelial cell sheet is free from the use of 3T3 feeder, whereas human oral mucosal epithelial cell culture is unstable without 3T3 feeder. In addition, serum use as a medium additive has problems as followings; animal derived serum has a possibility to transmit unknown infection and lot differences, patient derived serum has issues of invasiveness of blood collection, a possibility of preparation mistake, and individual differences on constituents. Therefore, there is a compelling need for the development of a 3T3 feeder- and serum-free culture system for oral mucosal epithelial cells, but this condition is still unstable and needs possible additional factors for sustaining the stable supplies of regenerative medicine products. This thesis aimed the elucidation and application of effective supplemental factors for stratified squamous epithelial cell culture.

Chapter 1 describes the backgrounds of regenerative medicine, stratified squamous epithelium, and the application and current issue of epithelial cell sheets.

Chapter 2 describes aims of this thesis.

In chapter 3, interspecific differences of in the *in vitro* behaviors of oral mucosal epithelial cell were clarified among human, rat, mouse, dog, rabbit, and pig. Human and pig epithelia were relatively thicker among 6 species. Colony forming efficiency of rat was the highest, and followed by the order of dog, human, rabbit, and pig. Colonies of mouse cells were all paraclone and uncountable in colony forming assay. Rabbit and pig cells showed a poor proliferation and no cell sheet forming ability without a feeder layer. On the other hand, even without a feeder layer, cultured dog and mouse cells formed contiguous cell sheets, and dog cell sheets were able to be produced steadily regardless of culture condition as long as the seeding cell density was high. These results indicated that interspecific variation was considerable in oral mucosal epithelial cells, and specific experimental animal or human cells must be chosen in accordance with the intended use. In a condition with both 3T3 feeder- and

cholera toxin-free culture medium, high cell density-seeded dog oral mucosal epithelial cells grew strongly. Moreover, dog cells showed a different inclination regarding the responsibilities for cholera toxin and cellular cAMP concentration.

In chapter 4, IL-1 receptor antagonist was identified as a possible growth factor by focusing on the outstanding proliferation ability of dog oral mucosal epithelial cell. Collected 48-h-incubated supernatant of dog oral mucosal epithelial cell culture promoted rat oral mucosal epithelial cell growth, and the growth effects were high by those collected on 5 day to 9 day. By the time-point analysis of growth factors on cultured dog oral mucosal epithelial cell, 14 growth factors, of which expressions were high on day 5 to day 9, were screened. A statistically significant promotive activity was observed in IL-1RA, and a significant inhibitory activity was observed in IL-1α. Moreover, IL-1RA and anti-IL-1α neutralizing antibody promoted normal human epidermal keratinocyte growth and induced a stem/progenitor cell marker and normal differentiation markers. Furthermore, fabricated human epidermal keratinocyte sheets showed the high expressions of stem/progenitor marker, the non-abnormal expression of proliferation marker, and the normal expression of proper differentiation marker, indicating that IL-1RA has a clinical usability.

In chapter 5, all-*trans* retinoic acid (ATRA), a retinol derivative, was identified to be one of the most important replacements of serum. Interestingly, fabricated stratified epithelial cell sheets cultured under a serum-free condition were found to be fragile, and showed no contraction and a poor stratification after detachment from culture surfaces, whereas the cell sheet fabricated under a serum-added condition showed a strong contraction, and a stratified and robust structure. Cell sheets fabricated in a cytochalasin D, an actin polymerization inhibitor, added condition and a sodium azide, an ATP synthesis inhibitor, added condition showed no contraction even in a serum-added condition. In addition, the inhibition of Rho kinase, which is the downstream of small GTPase Rho, the inhibition of myosin ATPase, the inhibition of myosin light chain kinase, and Ca^{2+} chelation suppressed the contraction. However, lysophosphatidic acid and thrombin, which are known as Rho activators, showed no effect on cell sheet contraction in serum-free condition. DNA microarray analysis revealed that the most significant difference was observed in retinol metabolism pathway between serum-added and serum-free conditions. ATRA induced epithelial cell proliferation, cell sheet contraction and stratification, and the recovery of structural fragility in a serum-free culture condition in a dose-dependent manner. Organization of E-cadherin, α1-catenin, β-catenin, desmoglein 1, and desmocollin 3

were notably higher under a serum-added or ATRA-supplemented condition than under a condition without both of serum and ATRA. Combined with the results, the contraction needed both actomyosin activation and cell-cell junction maturation, which would be regulated by ATRA. Therefore, ATRA was able to be considered as one of the most significant replacement factors for serum.

In chapter 6, we clarified an epithelial proliferative effect by calpain inhibition. Because the study of chapter 4 found a suppressive activity on epithelial cell growth by IL-1α, we investigated an epithelial cell growth activity by the suppression of calpain, which makes mature and secretory IL-1α from pro-IL-1α by its proteinase activity. Attached cell number was significantly high, cell morphology was kept in uniform, and the cell proliferation was strongly enhanced in a calpain inhibited condition. In addition, no effect was observed in a condition with calpain inhibitors having huge molecular weight. Moreover, not only IL-1α expression was suppressed in the culture supernatant, but also mRNA expression and the pro-and mature-IL-1α level of the cells. These results indicated that calpain inhibitor induces epithelial proliferation partly by inhibiting IL-1α expression inside the cells, and the proliferation effect was found to be useful as an additional factor for epithelial culture.

In chapter 7, IL-1RA- and ATRA-added serum-free chemically defined medium was prepared. Cultured stratified squamous epithelial cells showed normal epithelial cell morphology and a proliferation activity, which were similar to those in a serum-added condition, indicating that a novel cell culture technology was developed for less-invasive and consistent regenerative medicine.

In chapter 8, the study conclusion and future prospect are described. From this study, effective factors for epithelial culture were elucidated.

This study would give (1) a reduction in the amount of collected tissue by biopsy from patients and (2) a possibility to shorten cell sheet fabrication time, indicating that these are great benefits for patients, medical workers, and regenerative medicine industries. Moreover, the findings would give a keen perception for proliferation, differentiation, stem/progenitor maintenance, and engineered tissue cytoskeleton of stratified epithelial cells. Furthermore, the comparable cell biological approach for growth factor exploration in chapter 4 can be applied to investigation for various growth factors.

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氏 名　　近藤　誠　　印

（２０１３年　７月　現在）

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