# －原著－

# 口腔粘膜上皮細胞培養上清で培養した口腔粘膜線維芽細胞の形質に関する検討

マザビンビンタモニル

新潟大学大学院医歯学総合研究科　口腔解剖学分野

# Characterization of an oral fibroblast phenotype cultured in oral keratinocyte-conditioned medium

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Key words: Oral fibroblast, oral keratinocyte, conditioned medium, phenotype, monolayer culture

**Abstract:**

Fibroblasts in monoculture grown in serum-containing medium are highly-proliferating. However, data suggested that use of static fibroblasts provides a better model to study biological phenomena than proliferating fibroblasts. Since previous studies have stated keratinocyte-conditioned medium reduced fibroblast proliferation, this study aimed to examine if the oral keratinocyte (OK) -conditioned medium (CM) can decrease oral fibroblasts (OF) proliferation and to characterize their phenotype. Primary human OK and OF were grown in a completed EpiLife® (0.06mM $Ca^{++}$) and Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum (DMEM-CS), respectively. OK-CM was conditioned for 24 hours in a near-confluent OK culture. OFs plated in a micro-plate well were cultured with DMEM-CS, serum-free DMEM (SF-DMEM) and OK-CM for up to 96 hours. Proliferation rate and cell cycle profile were analyzed using a MTT assay and a fluorescence-activated cell sorter. The "phenotypic changes" of OFs were determined by the activity of senescent-associated $\beta$-galactosidase ($\beta$-gal) and the secreted protein levels including keratinocyte growth factor (KGF), human type I collagen and matrix metalloproteinase-1 (MMP-1) measured by enzyme-linked immunosorbent assay (ELISA). The proliferating rate and the proportion of cells in S phase were significantly lower when cells were cultured in OK-CM. The $\beta$-gal activity suggested OFs in OK-CM still had proliferating potential. ELISA assay showed OFs cultured in OK-CM produce KGF and MMP-1 as did OFs grown in DMEM-CS while their ability to produce type I collagen was significantly lower than OFs in DMEM-CS. This study suggested the OK-CM generated a quiescent OF population possessing the characteristic of extracellular matrix degradation rather than synthesis.

MTT : 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

日本語要旨：

生体反応の研究に用いる線維芽細胞は増殖している細胞ではなく休止期のものが好ましい。上皮細胞の培養上清に線維芽細胞の増殖抑制効果があることが報告されている。本研究の目的は口腔粘膜上皮細胞の培養上清が培養口腔粘膜線維芽細胞の増殖性を抑え，細胞形質を変化させるかを検証することである。ヒト初代口腔粘膜上皮細胞（OK）と線維芽細胞（OF）はそれぞれ EpiLife と 10%ウシ胎児血清含有ダルベッコ改変イーグル培地（DMEM）（DMEM-CS）中で培養した。コンフルエントに近い口腔粘膜上皮細胞を 24 時間培養したものを上清とした。口腔粘膜線維芽細胞は DMEM-CS，DMEM 培地単独（SF-DMEM），口腔粘膜上皮細胞培養上清（OK-CM）の 3 種類の培地で，最長 96

時間培養した。細胞増殖能と細胞周期はそれぞれMTT assayとセルソーターで解析した。細胞形質変化の分析はさらに，βガラクトシダーゼ活性発現とエライザ法（ELISA）による各種タンパク質（ケラチノサイト増殖因子（KGF），タイプⅠコラーゲン，マトリックスメタロプロテアーゼ-1（MMP-1））分泌量で検討した。細胞増殖能とS期の細胞の割合は有意にOK-CMで培養した細胞で低下しており，G0/G1期停止による細胞増殖抑制の可能性が示唆された。またOK-CM培養では，βガラクトシダーゼ活性を示した細胞は少数で，口腔粘膜線維芽細胞は休止期にいるが，増殖能が失われていないことを示した。KGFとMMP-1の産生量は，DMEM-CSで培養した細胞と同等であったのに対し，Ⅰ型コラーゲンの産生量が有意に低かった。本研究から，OK-CMは培養口腔粘膜線維芽細胞の増殖能を抑え，かつ細胞外基質分解性の細胞形質となることが示唆された。

## Introduction

Constant tissue renewal of stratified squamous epithelia such as skin and oral mucosa is maintained by a balance between cell differentiation and desquamation at the epithelial surface and the replenishment of keratinocytes by cell division occurred in basal and parabasal cell layers[1)] . Thus, mitotic activity of keratinocytes is frequently recognizable *in vivo*. In contrast to keratinocytes, fibroblasts in normal adult skin and oral mucosa infrequently divide and are static in nature[2,3)] . In addition, in an uninjured tissue, they are relatively inactive in terms of protein synthesis and thin in histological appearance[4)] . However, the wound healing process induces phenotypic alterations in the resident fibroblast population from quiescence to migration and proliferation, contracting cells and producing extracellular matrix[5)] . Since fibroblasts in a monoculture are invariably highly proliferative in a serum-containing medium, this fibroblast phenotype seems to be "activated" fibroblasts in vivo in response to wound healing[4)] . While studies have used proliferating fibroblasts in a monoculture to examine the cytotoxic effects of pharmaceuticals and chemicals and consequent cellular responses[6,7)] , data obtained from those experiments cannot be extrapolated to the effects in the target tissue in vivo because their cellular responses may be different from those of inactive fibroblasts, similar to the phenotype in an uninjured tissue.

Recently, researchers have paid an attention to tissue engineering as an emerging technology for regenerative medicine. In contrast to a monolayer culture system, it is well-known that cells cultured in a three-dimensional (3D) scaffold behave differently[8,9)] . In fact, the proliferation rate and collagen production by fibroblasts embedded in a 3D collagen matrix decreased[10,11)] . Those fibroblast phenotypic changes were confirmed to be beneficial to dermal tissue regeneration[12)] . Although most products commercially available such as Dermagraft® and Apligraf® incorporate normal proliferating early passaged fibroblasts from foreskin to fabricate the tissue-engineered substitutes, there is a body of data suggesting that use of static fibroblasts is a better model to address biological phenomena than primary proliferating fibroblasts even when fabricating 3D tissue-engineered constructs[5)] . Thus, to implement a better model, we should use different culture technique that allows to provide quiescent fibroblasts even in a monolayer culture system.

There have been two means to modulate fibroblast phenotype to a static and non-proliferating nature mimicking residential fibroblasts in dermal tissue. One is utilization of irradiated dermal fibroblasts[13)] , and the other is use of keratinocyte-conditioned medium (CM)[14)] . Previous reports stated skin keratinocyte-CM reduced skin fibroblast proliferation as well as collagen matrix synthesis[2,15,16)] . In the oral mucosa, interactions between keratinocyte and fibroblast are also critical to basic research as a tool to translate into regenerative medicine. However, there have been few studies on their cross-talk by using oral keratinocytes-conditioned medium (OK-CM)[17)] . Consequently, we are interested in the feasibility of OK-CM that is always discarded in our laboratory.

In this study, we hypothesized the OK-CM yielded in our laboratory might contribute to oral fibroblast (OF) phenotypic changes from an actively-cycling state to a more static state. Thus, this study aimed to examine if the OK-CM can decrease OFs proliferation in a monolayer culture. In addition, we further characterized the phenotypic changes of OFs cultured in OK-CM. The re-use of OK-CM could provide an easier and inexpensive approach for studying the cellular biology of OFs.