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Modulation of Oral Epithelial Cell Properties by Insulin

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Abstract

The cell culture environment regulates epithelial cell properties by changing a number of signaling pathways and intercellular interactions among different cell types. Culture medium contains many components that stimulate cell phenotypic changes. Many studies have reported modulating cells by altering the culture environment. Changes in culture conditions can induce the epithelial to mesenchymal transition and the mesenchymal to epithelial transition. Therefore, microenvironmental interactions between the culture medium and cells entail cytoskeletal remodeling. We studied the characteristics of oral epithelial cells according to insulin concentration, which is used as a cell culture supplement because it delays apoptosis and promotes proliferation. A high insulin concentration turned fibroblastic epithelial cells into a cobblestone-like morphology, shortened cell-to-cell distance, and contributed to increase cell growth. These results provide data on the effect of insulin in oral epithelial cells under in vitro culture conditions.

Keywords: EMT, Environment, Insulin, MET, Morphology

1. Introduction

Culture conditions are the cellular microenvironment that determines the dynamic characteristics of epithelial cells[1]. Different components are used in epithelial cell culture for cell productivity. However, changing the culture microenvironment can produce different cellular phenotypes[2]. The tumor microenvironment is affected by different factors secreted by the interactions between cells and diverse nutritional components[3]. Thus, an altered tumor microenvironment impacts tumorigenesis.

Keratinocytes are influenced by a dynamic microenvironment and modulate proliferation, differentiation, and morphology[4]. Culturing human keratinocytes in different growth media shows that the microenvironment affects keratinocyte properties. A 3T3 feeder layer or fibroblast conditioned medium are used to proliferate keratinocytes[5]. In addition, serum-free media or low calcium serum-free media are used for keratinocytes in culture[6,7]. These different culture methods not only affect proliferation but also differentiation and morphology.

Epithelial cells can transform into motile mesenchymal cells, which assume a more extended and elongated shape. This modification causes loss of intracellular adhesion and has been defined as the epithelial to mesenchymal transition (EMT)[8]. The mesenchymal to epithelial transition (MET) enables epithelial-like cancer cells to colonize and mature at remote sites to form metastases[9]. It has been suggested that the EMT is an early stage process in carcinogenesis, whereas the MET occurs at a later stage for metastasis[10]. These findings have been confirmed as a mechanism related to the cancer microenvironment; however, little is known about the relationship between the EMT and MET due to the culture microenvironment of Immortalized Human Oral Keratinocytes (IHOKs) until now.

We investigated the influence of insulin, which is used as a cell culture supplement, on IHOKs. Insulin is

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widely used in mammalian cell culture because it delays apoptosis and promotes cell proliferation[11].

Three media with different insulin concentrations were used to investigate the effect of insulin on IHOK culture. IHOK characteristics, including cell morphology, proliferation, and adhesion were examined after insulin treatment. As result, higher insulin concentrations lead to an increase in the number of cells that changed to an epithelial cobblestone-like morphology from an elongated shape, and cell-to-cell adhesion increased. In addition, relative growth rate of IHOKs was highest when the cells were cultured in medium with the highest insulin concentration (50 μg/μl).

Taken together, a multifaceted exploration of culture conditions is needed because cell culture components such as insulin highly affected cellular properties.

2. Methodology

2.1 Reagents and Antibodies

Antibodies against E-cadherin and snail were obtained from Cell Signaling Technology (Danvers, MA) and β-actin was purchased from Sigma (St. Louis, MO, USA).

2.2 Cell Culture

IHOK was maintained in EF-medium. EF-medium was consist of Dulbecco's Modified Eagles Medium (DMEM; Gibco BRL, USA) and Ham's Nutrient Mixture-F12 (Gibco BRL, USA) culture medium at a ratio of 3:1(P media), supplemented with 10% Fetal bovine serum (FBS), 1% penicillin/streptomycin, 0.01 μg/ml cholera toxin, 0.04 μg/ml hydrocortisone, 0.5 μg/ml insulin, 0.5 μg/ml apo-transferrin, and 0.2 μg/ml 3'-5-triodo-l-thyronine (these supplements were purchased from Sigma St. Louis, MO, USA). For 30 day, IHOK was cultured in medium including different concentration of insulin as control (0.5 μg/ml), 25μg/μl, and 50μg/μl.

2.3 Immunoblotting

Cells were washed with cold PBS and lysed in Cell Lysis Buffer (Cell Signaling Technology). After lysis, protein complexes were boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer and separated by 10% SDS-PAGE. Then, proteins were transferred to polyvinylidene fluoride membranes, and the membranes were blocked in 5% milk in Tris-buffered saline containing tween 20 and then immunoblotted with appropriate primary antibodies. Blots were then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Cell Signaling Technology), and detection was done by chemiluminescence (Santa Cruz Biotechnology).

2.4 Cell Proliferation Assay

The proliferation of IHOKs which were cultured in the media including different concentration of insulin for 30 days was measured by 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 2000 cells were seeded in each well of 24-well plates and MTT solution was added to each well. After the plate was incubated at 37°C for 4 hr, the medium was removed, and 200 μl of DMSO was added per well. Absorbance was measured for up to 72 hr at 540 nm using the microplate reader (Bio-Rad Laboratories, Hercules, CA).

2.5 Statistical Analysis

To present a significant difference between control and experimental groups in cell proliferation assay, Mann-whitney test (SPSS Inc, IBM, Chicago, US) were used. The significant difference in results was determined as $P < 0.05$.

3. Findings

3.1 Insulin Changes the Cell Morphology of IHOK

Control IHOK cells were cultured in medium containing 5μg/μl of insulin, and they indicated fibroblastic morphology without cell to cell adhesion. To examine the changes of IHOK cell characteristics according to insulin concentration, IHOK cells were cultured for 30 days in media containing two different concentrations of insulin, 25 μg/μl and 50 μg/μl, respectively. After culturing for 30 days, the IHOK cells displayed changes in morphology. At the insulin concentration of 25 μg/μl, the acicular control IHOK cells were changed to foliate appearance and the cell to cell space became narrow. At the concentration of 50 μg/μl, IHOK cells had epithelial cobblestone-like morphology, and cell to cell contact was observed (Figure 1). These results indicate that insulin affects epithelial cell morphology in a concentration-dependent manner.

For 30 day, IHOK was cultured in medium including different concentration of insulin. Control IHOK

was cultured in medium including 5 μg/μl of insulin (left panel). IHOK of middle panel was cultured in media including 25 μg/μl of insulin. The medium of IHOK of right panel contained 50 μg/μl of insulin.

3.2 Culture Conditions Induced Epithelial and Mesenchymal Differentiation in IHOKs

To investigate whether the morphology changes of IHOK cells were related to MET, MET markers proteins were examined. The expression of E-cadherin, an epithelial differentiation marker, was increased according to the addition of insulin. In addition, the expression of SNAIL, a mesenchymal differentiation marker, was decreased in the IHOK cells cultured in 25 μg/μl of insulin. However, the IHOK cells cultured in 50 μg/μl of insulin did not show a particular difference compared to the control cells (Figure 2). Though SNAIL expression was not decreased at 50 μg/μl of insulin, these results show alteration of epithelial differentiation markers and mesenchymal differentiation markers according to the insulin concentration.

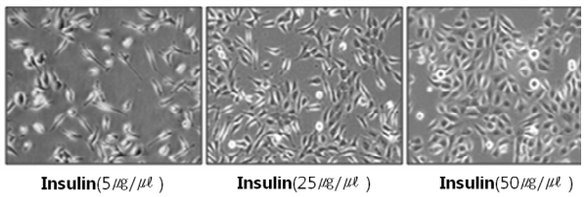

Figure 1. Culture of IHOK according to Insulin Concentration.

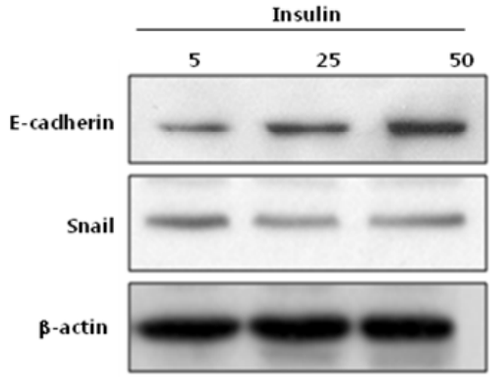

Figure 2. Alteration of Protein Expression by Insulin Concentration.

IHOKs cultured in different insulin concentration for 30 days were examined for expression of MET proteins. Western blot was taken to assess the protein expression levels of E-cadherin, the epithelial markers, and snail, the mesenchymal markers.

3.3 Insulin Affects Cell Growth

Next, we compared the proliferation rate with the concentration change of insulin. Control IHOK and IHOK cells with altered properties after 30 days at different insulin concentrations were inspected by MTT assay for 3 days at each insulin concentration. The relative growth rate was increased according to the rise of insulin concentration (Figure 3).

IHOKs, which were changed in three different concentration of insulin, were cultured in each culture media for 3 days. Cell growth rate was measured by MTT assay using 24 well plates. Each assay was performed in triplicate ($^{*}P < 0.05$).

Taken together, these results indicate that insulin as culture supplement affects properties of epithelial cells; therefore, in study using cell culture, the deliberation of culture microenvironment is indispensible.

This study demonstrated the properties of epithelial biology through modulation of IHOK phenotypes by change of culture microenvironment. Besides IHOK, several studies on the phenotype changes of cancer cells and fibroblasts in different culture conditions have been reported. For example, terminal differentiation was related to human squamous cell carcinoma[12], and morphological and biochemical changes were associated with culture conditions in human small cell lung cancer[13].

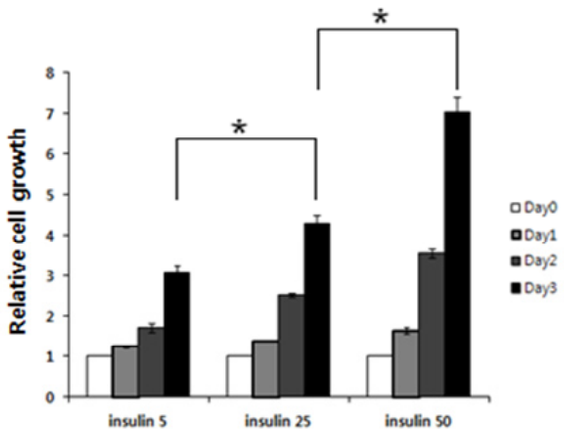

Figure 3. Effect of Insulin Concentration in Cell Growth.

In addition, alterations of the phenotype, morphology, and drug sensitivity of colon cancer cell lines were examined under various culture conditions[14], and a comparison of cell morphology and metabolic activity was reported in prostate cancer cell culture[15]. The protein profile of human dermal fibroblasts was also investigated according to culture conditions[16].

Cellular differentiation is the process by which a less specialized cell is changed into a more specialized cell type. Differentiation dramatically changes cell size, shape, membrane potential, metabolic activity, and responsiveness to signals. These alterations largely come from highly controlled modulations in gene expression. The terminal differentiation of epidermal keratinocytes involves dynamic morphological changes, including drastic cytoskeletal rearrangement[17].

In the present study, we investigated the alteration of epithelial properties due to insulin, which is used as a supplement of cell culture because it delays apoptosis while promoting the proliferation of cells. Control IHOK, whose insulin concentration was 5 μg/μl, presented fibroblast-like spindle morphology. However, IHOK cells were changed to epithelial cobblestone-like morphology in a concentration-dependent manner. Among cell phenotype changes due to insulin, the change from fibroblastic morphology to epithelial appearance due to insulin in granulosa cell culture was studied in 1981, and it was explained that immature granulosa cells showed fibroblastic morphology in vitro, whereas mature granulosa cells presented epithelial morphology[18]. Moreover, the cobblestone-like cells observed herein which were changed due to insulin displayed decreased expression of vimentin, a mesenchymal differentiation marker, and increased expression of E-cadherin, considered as an epithelial controller. In addition, the increase of IHOK proliferation due to the increase of insulin concentration has been supported in several studies[19,20]. Therefore, IHOK cells may have undergone differentiation for epithelial maturation due to the presence of insulin.

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