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Laminin peptide YIGSR enhances epidermal development of skin equivalents

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ABSTRACT

Since the use of animal experimentation is restricted with regard to cosmetic materials, alternative *in vitro* models such as skin equivalents (SEs) are needed. Laminin is one of the major non-collagenous glycoproteins. The pentapeptide YIGSR (Tyr-Ile-Gly-Ser-Arg) is a functional motif of laminin that binds to the laminin receptor. In the present study, we examined whether YIGSR could improve the reconstruction of SEs. YIGSR has no effects on monolayer cell proliferation of CCD25-Sk fibroblasts or HaCaT keratinocytes. Interestingly, YIGSR decreased TGF-β1 levels, although it promoted type I collagen synthesis in CCD25-Sk cells. In HaCaT cells, YIGSR decreased the expression of involucrin and loricrin, which are differentiation markers. Furthermore, YIGSR increased levels of proliferating cell nuclear antigen (PCNA), p63, and integrin α6, and decreased involucrin in SE models. In addition, two models containing YIGSR (mixed with dermal equivalents or added into media) did not show any differences in expression levels of PCNA, p63, integrin α6, and involucrin. Therefore, YIGSR is a useful agent for reconstruction of SEs, independent of its method of application. These results indicate that YIGSR stimulates epidermal proliferation and basement membrane formation while inhibiting keratinocyte differentiation of SEs. Taken together, these results indicate that YIGSR promotes the reconstruction of SEs, potentially via decreased TGF-β1 levels and consequent inhibition of epidermal differentiation.

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1. Introduction

As the cosmetic market has grown, interest in novel cosmetic materials has also increased. Previously, safety testing using animals was considered essential before releasing a product. However, the European Union banned animal testing of cosmetics because of animal-rights concerns. Consequently, alternative *in vitro* methods are needed. Various *in vitro* tests have been employed, including microbiological tests and cell culture tests using human keratinocytes or fibroblasts seeded in monolayers [1]. Recently, a skin equivalent (SE) containing a dermal equivalent (DE) has emerged as a useful alternative testing model. The DE is an artificial dermis of skin constructed by mixing fibroblasts and collagen gel. The SE is a form of artificial skin containing epidermal layers and dermis constructed by seeding human keratinocytes onto a DE to produce a fully differentiated epidermis [2]. The reconstruction of SEs also facilitates research in dermatology and skin pharmacology.

In human skin, the extracellular matrix (ECM) plays a pivotal role in regulating cell proliferation, migration, and adhesion in tissues. There are many different ECM proteins, such as collagen, fibronectin, elastin, and laminin, among others. Collagen type I accounts for approximately 25–30% of all proteins in the body, is the main component of dermal skin, and is the major protein comprising the ECM [3]. Collagen is synthesized in fibroblasts and is regulated by various factors [4]. Laminin is a major non-collagenous glycoprotein that acts as an important peptide component of base membranes, and promotes cell growth, migration, adhesion, and differentiation [5–7]. Laminin forms a cruciform structure composed of three chains, α, β1, and β2, which

Abbreviations: BM, basement membrane; DE, dermal equivalent; ECM, extracellular matrix; PCNA, proliferating cell nuclear antigen; SE, skin equivalent.

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are linked covalently by disulfide bonds [8,9]. The pentapeptide YIGSR (Tyr-Ile-Gly-Ser-Arg) in the laminin β1 chain is a functional motif that binds to the laminin receptor [10]. YIGSR has been reported to facilitate cellular attachment and help bear mechanical stress in silicone membranes [11]. In a previous study, YIGSR was found to have effects on collagen synthesis in human dermal fibroblasts [12]. In the skin, the composition of the dermis can affect the epidermis. Therefore, in the present study, we examined whether YIGSR influences SEs.

Studies in many cell types have shown that proliferation and differentiation are inversely correlated processes [13,14]. Recent work has made it clear that cell proliferation and differentiation are regulated simultaneously but independently, that cells often start differentiating long before they stop dividing, and that activation of differentiation is not limited to any particular segment of the cell cycle [13]. Thus, we investigated the relationship between proliferation and differentiation in an SE model.

2. Materials and methods

2.1. Materials

YIGSR peptides were obtained from ANYGEN Co. (Gwangju, Korea). Powdered Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 nutrient mixture (F-12) powder, L-ascorbic acid, formaldehyde, sodium bicarbonate, HEPES, hydrocortisone, isoproterenol, involucrin antibody (I9018), and insulin were obtained from Sigma (St. Louis, MO, USA). Recombinant human epidermal growth factor (EGF) and Fetal Bovine Serum (FBS) were purchased from Invitrogen Co. (Gibco, Camarillo, CA, USA). DMEM/Nutrient Mixture F-12 in a 3:1 mixture, sodium hydroxide, trypsin-EDTA, and antibiotic-antimycotic mix (penicillin, streptomycin) were purchased from WelGENE (Daegu, Korea). Specific antibodies for type-1 collagen (20111) were obtained from Novotec (Lyon, France). Anti-TGF-β1 antibody (ab64715) was obtained from Abcam (Cambridge, MA, USA). Antibody against actin (sc-1616) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Loricrin antibody was obtained from Thermo Fisher Scientific (Rockford, IL, USA). Secondary antibodies specific to anti-goat IgG (PI-9500), anti-mouse IgG (PI-2000), and anti-rabbit IgG (PI-1000) were purchased from Vector Laboratories (Burlingame, CA, USA).

2.2. Cell culture

The human fibroblast cell line CCD-25Sk was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA), and the human keratinocyte cell line HaCaT was purchased from Cell Lines Service (Eppelheim, Germany). The cells were grown in DMEM supplemented with 10% FBS, 50 μg/ml penicillin, and 50 μg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere.

2.3. EZ-Cytox cell viability assay for cell proliferation

An EZ-Cytox cell viability assay kit (iTsBio; Seoul, Korea) was used to measure cell proliferation. Both HaCaT cells and CCD-25Sk cells were seeded onto 24-well plates (1×10^4 cells/well) and cultured in DMEM containing 10% FBS at 37 °C in 5% CO_2. Twenty-four hours later, media was removed and both cell lines were treated with various concentrations of YIGSR peptides in serum-free DMEM at 37 °C in 5% CO_2 for 72 h. We then added 100 μl of kit reagent to each well and incubated the cells for 1 h under the same conditions. We measured the absorbance at 450 nm using an ELISA reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA).

2.4. Western blot analysis

For western blot analysis, a total of 1.5×10^5 CCD-25Sk cells or 3.5×10^5 HaCaT cells were seeded on 100-mm dishes. Both cell types were lysed in cell lysis buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Complete™, Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA). Proteins in the cell lysates were separated by SDS-polyacrylamide gel electrophoresis using 10 μg of protein per lane, blotted onto polyvinylidene fluoride (PVDF) membranes, and blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20. Blots were then incubated with appropriate primary antibodies at a dilution of 1:1000 and then with horseradish peroxidase-conjugated secondary antibodies. Bound antibodies were identified using an enhanced chemiluminescence testing kit (Thermo Scientific Inc., Bremen, Germany). All of the immunoblot images were observed using an LAS-1000 lumino-image analyzer (Fuji Film, Tokyo, Japan).

2.5. Preparation of dermal equivalents

In brief, type-1 collagen extracted from rat tail tendons was dissolved by stirring in 1/1000 glacial acetic acid at 4 °C for a week. DEs were made by mixing the following solutions: eight volumes of 1% type I collagen solution, one volume of 10 × reconstitution buffer (0.26 mM $NaHCO_3$, 0.05 N NaOH, 200 mM HEPES), and one volume of 10 × medium (DMEM: F-12, 3:1). We created three DE models: control, 1 μM YIGSR added to DE, and 1 μM of YIGSR added in the media. All of the DEs contained 3×10^5 CCD-25Sk cells. We poured 3 ml of each mixture into 24-mm transwell inserts with 3.0-μm pore polycarbonate membranes (Corning, Inc., Corning, NY, USA). The inserts were then placed in the incubator at 37 °C to gel.

2.6. Reconstruction of skin equivalents

To reconstruct SEs, 1×10^6 HaCaT cells were seeded onto DEs and cultured under submerged conditions for 24 h, and then at the air-liquid interface for 12 days. The growth medium consisted of DMEM/F-12 supplemented with 5% FBS, 0.4 μg/ml hydrocortisone, 1 M isoproterenol, 5 μg/ml insulin, and 25 μg/ml ascorbic acid. Epidermal growth factor (EGF) was added to the growth medium at 1 ng/ml for the submerged culture, and at 10 ng/ml EGF for the air-liquid interface culture. The medium was replaced with fresh medium three times per week. YIGSR was added when changing media.

2.7. Immunohistochemistry

After 13 days, SEs were fixed in 10% formaldehyde for 1 day and processed for conventional paraffin embedding. Four-to six-μm-thick sections were prepared. For morphologic analysis, sections were stained with hematoxylin and eosin (H&E). For immunohistochemical analysis, sections were processed using the avidin-biotin-peroxidase complex technique (DAKO, Glostrup, Denmark). Antibodies specific to p63, proliferating cell nuclear antigen (PCNA), and α6-integrin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); involucrin (I9018) was from Sigma-Aldrich, Inc.; and type I collagen (No. 20111) was from Novotec. Section images were taken using an Olympus BX51 microscope (Tokyo, Japan) equipped with a digital camera (CC 12 Soft Imaging System, Olympus).

2.8. Statistics

Intergroup differences were assessed by analysis of variance

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