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The transdermal inhibition of melanogenesis by a cell-membrane-permeable peptide delivery system based on poly-arginine

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ABSTRACT

Topical therapy is the most favored form of treatment for whitening against hyper-pigmentation and sunburn because it lends itself to self-administration, patient compliance and an absence of systemic adverse effects. However, high-molecular-weight, hydrophilic chemicals are difficult to use as transdermal delivery drugs and the use of topical drugs has been highly limited. There are now many potent tyrosinase inhibitors, for example, sulfite or kojic acid, but the efficacy of their skin transduction remains a big problem. Furthermore, melanogenesis inhibitors from natural sources have great potential, as they are considered to be safe and largely free from adverse side effects. We applied 11-arginine (11R), a cell-membrane-permeable peptide, as a transdermal delivery system with a skin delivery enhancer, pyrenbutyrate. We performed intracellular screening for melanogenesis inhibitors with 11R fused with several kinds of tyrosinase inhibitory peptides from natural sources. Of 28 tyrosinase peptides, 13 melanin synthesis inhibitory peptides were selected. Peptide No. 10 found in gliadin protein, a wheat component, most strongly inhibited melanin production. This No. 10 peptide, of only 8 amino acids, fused to 11R showed no cytotoxicity and inhibited melanin synthesis as determined through melanin content measured using an absorption spectrometer and observation with a transmission electron microscope. Next, we transduced this 11R-No. 10 into skin with an 11R transdermal delivery system after previous treatment with pyrenbutyrate and performed daily repetitive topical application for two weeks against a UV-induced sun-tanning guinea pig model. We observed a whitening effect in a model skin sample by Masson-Fontana staining and the 11R-No. 10 peptide-applied area showed significant melanogenesis inhibition. These results show that 11R using a transdermal drug delivery system with melanogenesis inhibitory peptide is a very safe and promising method for applications from cosmetics to the pharmaceutical industry.

1. Introduction

The skin, which consists of several layers including the stratum corneum (SC), epidermis and dermis, is the primary defense system, maintaining body temperature, blocking ultraviolet radiation and providing substantial protection from pathogens via the SC. The SC is composed of corneocytes interspersed in a laminate of compressed keratin and intercorneocyte lipid lamellae; it functions to conserve water and electrolytes, and is selectively permeable to certain substances such as drugs [1].

In the percutaneous Drug Delivery System (pDDS), the biggest problem for transdermal absorption is conquest of the SC barrier. Almost no materials that are water-soluble and have a molecular weight of more than 500 Da can pass through the SC [2]. However, several delivery systems that can enhance drug permeation through the skin have been developed recently. The pDDS method of a drug is currently classified roughly into chemical promotion and physical promotion [3]. In chemical promotion, liposome is used as a minute capsule having a particle size of 100 nm composed of phosphatides. It can enclose various molecules and seems to be an ideal drug delivery system (DDS) for

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physiologically active substances because of its superior biocompatibility and biodegradability [4]. However, the liposome skin transduction method depends on the polarity and molecular structure of the drugs, and cannot be applied to all drugs. In a chemical transdermal system with peptide, Candan et al. [5] reported that water-soluble hydroquinone and high-molecular-weight enhanced green fluorescent protein fused to cell membrane-permeable peptide (CPP, 11R), with a tissue-enhancing agent, pyrenbutyrate, could penetrate the SC barrier, enter the dermis and epidermis cells and show a whitening effect against a guinea pig sun-tanning model. They also reported that whitening agent fused to CPP can be applied in cosmetics to pharmacological applications safely and effectively. Physical skin promotion methods include iontophoresis [6] and micro-needle [7]. However, these two are non-physiological pDDSs beyond the SC barrier and need further improvement of the associated devices for their application.

Upon application of an agent outside the skin by a chemical method, two ways of penetrating the SC barrier have been reported: the appendage route, which involves hair follicles and sweat glands and the SC route, which involves intracellular space and transcellular penetration of SC in percutaneous drug delivery during skin absorption. In terms of pDDS through the appendage route, appendage delivery through follicles and sweat glands partly contributes to topical delivery, but the proportion of appendage to the skin surface area is less than 0.1%. In addition, several papers reported that drugs with a molecular weight over 500 Da or polar material could not enter the skin through the barrier of the SC. SC is the greatest barrier against skin transmission. Two transdermal delivery routes, the transcellular route going through keratin cells in the path via the SC and the intracellular route via the intercellular space, are the main paths for topical absorption. Therefore, conquest of the SC barrier is the biggest problem for transdermal introduction [2,8,9]. To overcome this barrier, numerous strategies have been developed, including chemical penetration enhancers and prodrugs [10]. However, whether these penetration-enhancing methods have the potential to control the drug delivery towards either transdermal absorption or cutaneous targeting remains unclear (Figs. 1–5).

Protein transduction system is a widely accepted method of delivering proteins, peptides, siRNA and biologically active compounds across the cell membrane by fusing with cell-penetrating peptides (CPPs) such as poly-arginine and the protein transduction domain of TAT [11–14]. This methodology attracted our attention not only as a means of cell biological study but also because of its potential for pharmaceutical vectors. CPP-fused proteins are rapidly internalized by lipid raft-dependent macro-pinocytosis. After internalization via the macropinocytotic pathway, the proteins are carried to macropinosomes, where most of them are then degraded [15]. For the control of hair follicular cell cycling to promote hair growth, only the protein transduction system was effective for inducing mouse hair in nude mice. However, recent studies have shown that the ability to cross lipid bilayers and gain access to the cell interior by CPPs, especially poly-arginine, is enhanced in the presence of the hydrophobic counteranion 4-(1-pyrenyl)-butyric acid (pyrenebutyrate, PB) [5] [8]. The negatively charged counteranions and high hydrophobicity of PB can exert a great influence on the translocation behavior of arginine peptides in artificial membranes. These features of the combination of poly-arginine and pyrenebutyrate are thought to be useful for the transdermal delivery of hydrophilic chemicals. PB works as a new skin delivery enhancer with poly-arginine peptide transdermal delivery.

In the field of the whitening of skin, one of the most important factors is to control tyrosinase activity and melanogenesis. In the present study, we attempt to establish a new intracellular peptide melanogenesis inhibitor screening method using a protein transduction system and established poly-arginine peptide transdermal DDS with a topical delivery enhancer, PB, and observe the whitening effect *in vitro* and *in vivo*.

2. Materials and methods

2.1. Peptide synthesis

Peptides were prepared by conventional Fmoc-based solid-phase peptide synthesis as described previously [16,17,18].

2.2. Cell lines and culture

B16 4A5 melanoma cell lines were cultured in D-MEM with 10% fetal bovine serum (FBS) and streptomycin at 100 μg/ml, at 37 °C in an atmosphere of 95% air and 5% CO_2 [19].

Mouse B16 melanoma cells (B16-4A5) were provided by the European Collection of Cell Cultures (ECACC). Cells were cultured in Dulbecco's modified Eagle's medium (D-MEM, Life Technologies, Grand Island, NY) with 10% fetal bovine serum (Life Technologies), 100 U/ml penicillin, 100 U/ml streptomycin and 0.2% L-glutamine (Life Technologies). Cultures were maintained at 37 °C in 95% air and 5% CO_2 in a humidified incubator.

2.3. Cell viability assay (WST-1 assay)

Cell viability was determined using the WST-1 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2, 4-disulfophenyl]-2H-tetrazolium, mono-sodium salt) assay (Roche Applied Science) as described previously. B16 4A5 cells (2×10^3) seeded on 96-well plates were cultured in D-MEM containing 10% fetal bovine serum and 1% penicillin and streptomycin for 24 h. After incubation for 72 h, cell viability was measured using the WST-1 assay according to the manufacturer's instructions (Roche Applied Science).

2.4. Measurement of melanin content

B16 4A5 melanoma cells (1×10^5 cells/well) were cultured in D-MEM medium for 24 h and then exposed to tyrosinase-inhibitor peptide at various concentrations ranging from 10 to 30 μM for 72 h. After retrieving the cells, they were washed with PBS and suspended in 0.4 ml of 1N NaOH at 80 °C for 2 h. The sample was then vigorously vortexed to solubilize the melanin pigment. The absorbance at 405 nm was measured using a microplate reader (Vient XS, DS Pharma Biomedical, Osaka, Japan) [20].

2.5. Transmission electron microscopy (TEM)

B16 4A5 melanoma cells (1×10^5 cells/well) were seeded on a 6-well plate and peptide was added (final concentration of 20 μM) after 24 h. The cells were exfoliated physically after 72 h and fixed with 0.1 M cacodylate buffer (pH 7.4) containing 2% glutaraldehyde (TAAB Laboratories Equipment, Berkshire, United Kingdom) and 2% paraformaldehyde (Wako) overnight. After fixing, samples were embedded in low-viscosity epoxy resin (TAAB Laboratories Equipment). The ultrathin sections were stained by uranium-lead double staining and observed using a transmission electron microscope (H-7650, Hitachi High-Technologies, Japan) [21].

2.6. Evaluation of the skin permeability using various TMR peptides

The hair from the back of a brown guinea pig (Female Weiser-Maples, SHIMIZU Laboratory Supplies, Kyoto, Japan) weighing 450–500 g was removed carefully using depilatory cream before the study. All procedures of animal experiments were approved by the Animal Ethics Committee of Okayama University (OKU-2013184). Areas of 4.0 cm^2 were marked on the dorsal trunk of the animals using a template. Skin permeability was examined using TMR-11R and TMR-FLAG. For topical treatment of peptides, PB (pyrenebutyrate) in propylene glycol [a mixture of 1 μl of PB (50 mM) and 99 μl of propylene glycol] was pre-applied on the skin. After 5 min, TMR-11R [a mixture of 1.25 μl of TMR-11R (20 mM) and 48.75 μl of propylene glycol] was applied to the same region. As a control, TMR-FLAG [a mixture of 1.25 μl of TMR-FLAG (20 mM) and 48.75 μl of propylene glycol] was applied on the skin of the same guinea pigs. Skin sections were obtained at 6, 12, 24 and 48 h after the topical applications with a 5-mm dermapunch (Maruho, Osaka, Japan). Excised skin samples were immediately frozen in Optimal Cutting Temperature compound (Sakura Finetek, Japan) and sequentially sectioned at a thickness of 10 μm. The sections were fixed with 4% PFA in 0.1 M phosphate buffer (pH 7.4) for 15 min. After washing with PBS, the sections were incubated with Hoechst 33342 (10 μg/ml) for 5 min, and viewed using a confocal microscope (FluoViewTM FV300, Olympus, Tokyo, Japan) [5].