Autoradiographic Verification of Transdermal Penetration of Oleic Acid-conjugated Peptide Nanosomes

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자가방사법에 의한 올레산이 결합된 펩타이드의 피부침투 확인

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ABSTRACT

Short peptides are potentially effective materials as cosmeceuticals, but their delivery across the skin can be problematic due to the ionic nature of peptides and the structure of the skin. For short peptide to be utilized as cosmeceuticals, its ability to penetrate the skin must be altered. In this study, we conjugated the widely used procollagen type I signal peptide, KTTKS, with oleic acid to improve the lipophilic properties of the peptide, and used the oleic acid-conjugated peptides to construct cosmeceutical nanosomes. Then we examined the penetration of cosmeceutical nanosomes prepared from isotope-labeled peptide into the skin after transdermal application using autoradiography. Because of its hydrophilic property of penta-peptide, the penta-peptide itself was not able to be penetrated through the stratum corneum of the skin. In contrast, nanosomes made from olecic acid conjugated penta-peptide were able to be penetrated through the stratum corneum effectively. Autoradiography showed the precise penetration points to dermal layer, demonstrating the appropriateness of this method for clarifying the mechanism of penetration of transdermal delivery systems.

Keywords : Autoradiography, Oleic acid-conjugated peptide, Transdermal penetration

INTRODUCTION

Most cosmetic products are utilized for adornment and are not categorized as drugs that alter cellular functions. Recently some of cosmetics have been developed to improve the skin physiology as anti-aging or moisturizing properties. These

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topical product formulations with drug-like benefits to the skin are called cosmeceuticals (Lupo & Cole, 2007). Topical cosmeceuticals is an appealing option for the delivery of medications, because the skin exhibits less enzymatic activity than other routes of administration, allowing delivered drugs to avoid first-pass degradation in the liver or gastro-intestinal tract (Benson & Namjoshi, 2008). Short peptides, which play an important role in modulating many physiological processes, have been studied for their potential cosmeceutical applications. Short peptides are beneficial for drug applications because of their small size, which makes them easily optimized and enables their therapeutic potential to be quickly investigated (Parthasarathi et al., 2008).

In cosmeceutical applications, the active form of peptides must be delivered to the target in a stable form. Although the topical use of peptides is potentially effective, delivery across the skin can be difficult due to the ionic nature of peptides and the structural properties of the skin. The skin has four main layers, stratum corneum (SC), epidermis (EP), dermis (D) and subcutis (S) (Young et al., 2006). The epidermis is a self-regenerating stratified squamous epithelium. The dermis is a layer of fibrocollagenous and elastic tissue that contains blood vessels, nerves and sensory receptors. The subcutis is the deepest layer of the skin. It is mainly comprised of adipose tissue, but also contains larger vessels that provide for delivery and return flow of blood (Young et al., 2006). The stratum corneum is a surface layer in direct contact with the external environment. The stratum corneum of the skin is composed of keratin and lipids, and thus prevents the penetration of various materials (Lademann et al., 2001; Schaefer & Redelmeier, 2006). Because stratum corneum prevents the penetration of materials, it is necessary to be designed for delivery of cosmeceuticals to their proper locations through stratum corneum.

After the cosmeceuticals design for skin penetration, it is necessary to be investigated whether it is delivered to proper location such as dermal layer through stratum corneum. A variety of imaging methods have been proposed to detect cosmeceuticals during skin penetration, including Fourier transform infrared attenuated total reflection (FTIR-ATR) spectroscopy, FTIR photo-acoustic spectroscopy (FTIR-PAS), confocal laser scanning microscopy (CLSM) and autoradiography (Alvarez-Rom et al., 2004; Gotter et al., 2008). CLSM and autoradiography have certain advantages when used to image the structure of skin. CLSM can confirm the penetration of fluorescent materials, but non-fluorescent materials must be labeled with a fluorescent moiety - a process that may alter the

structure and properties of the materials. Autoradiography also requires the isotope labeling of the materials, but the isotope labeling process, unlike fluorescent labeling, does not change the properties of the material. Furthermore, isotope-labeled materials can be distinguished from endogenous materials which are existent in the tissue itself. In addition, post-autoradiography staining can be used to display the current structure of the skin, showing the clear separation of skin layers. Thus, autoradiography can be used to confirm the penetration of artificially manufactured materials that can be contained endogenously, such as short peptides.

The most popular signal peptide used for various cosmeceutic peptide applications is the sequence, lysine-threonine-threonine-lysine-serine (KTTKS), found in type I procollagen. This pentapeptide has been demonstrated to stimulate feedback regulation of new collagen synthesis and increased production of extracellular matrix proteins, such as types I collagen, type II collagen and fibronectin (Katayama et al., 1993; Tsai et al., 2007). Various methods have been proposed for improving the delivery of cosmeceutical peptides to the dermis layer. Among them are fatty acid derivatives, which are used to increase the lipophilic property of the peptide, and KTTKS, which is linked to palmitic acid to enhance peptide delivery (Robinson et al., 2005).

In this study, we synthesized KTTKS and conjugated it with oleic acid to improve its lipophilic properties. From the oleic acid-conjugated peptide, we constructed cosmeceutical nanosomes. The penapeptide was labeled with isotope during synthesis and its penetration into the skin after transdermal treatment was examined. Penetration was assessed using autoradiography, which is capable of showing the structure of the skin and confirming the layer of penetration.

MATERIALS AND METHODS

1. Materials

Developing solution (Kodak), diisopropylcarbodiimide (Sigma), emulsion solution (RPN41, GE Healthcare), 1-[4,5-^{3}H] lysine monohydrochloride (GE Healthcare), oleic acid (Sigma), phenol (Sigma), TFA (sigma), triisopropylsilane (Sigma).

2. Methods

1) Preparation of oleic acid-conjugated KTTKS peptide
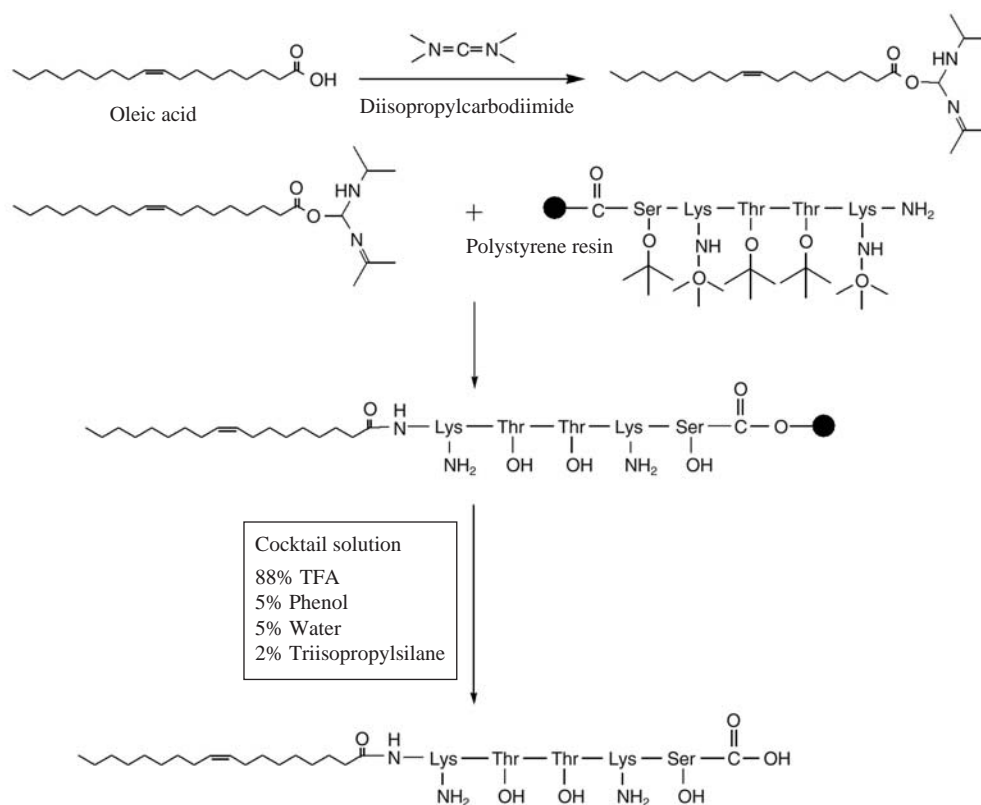KTTKS peptide was synthesized using a Perceptive Biosys-

Fig. 1. The reaction scheme for conjugation of synthetic peptide with oleic acid.

tems Pioneer Peptide Synthesizer with standard Fmoc (fluorenyl-9-methoxycarbonyl) pentafluorophenyl chemistry, and purified via high-performance liquid chromatography (HPLC). The resin-bound peptide was conjugated with oleic acid as shown in Fig. 1. Briefly, 2 mL of 250 mM oleic acid and 250 mM diisopropylcarbodiimide was prepared in DMF by stirring for 1 hr, and was mixed for 24 hr with 2 mL of 25 mM synthetic peptide in DMF. Then, oleic acid-conjugated peptide was purified by centrifugation at 2,000 rpm for 2 min. To remove the protection group and polystyrene resin from the conjugated peptide, the peptide was treated with 10 mL cocktail solution of 88% TFA, 5% phenol, 5% water, 2% triisopropyl silane for 2 hr and filtered with glass wool. The oleic acid-conjugated peptide was purified by precipitation in ether at $-80°C$ for 4 hr and centrifugation at 3,000 rpm for 5 min. After removing ether with N_2 gas, the oleic acid-conjugated peptide was analyzed by mass spectrometry.

2) Nanosome preparation

The oleic acid-conjugated KTTKS peptide was dissolved in phosphate buffer (pH 7.0), and oleic acid was added at the same ratio. The mixed solution was then vortexed, and the solution was passed through the microfluidizer (Emulsiflex C-30) 5 or 10 times. Their size was measured with an ELS (electrophoretic light scattering spectrophotometer) and cryo-TEM (cryotransmission electron microscopy).

3) Autoradiography

^{3}H-KTTKS peptide was synthesized with 1-[4,5-^{3}H]lysine monohydrochloride and then conjugated with oleic acid. Nanosomes were prepared from oleic acid conjugated ^{3}H-KTTKS. Isotope-labeled compounds were applied on the back of a hairless mouse (HR-1, SLC Japan) and left for 10 and 45 min in order to allow the compounds to penetrate the skin. Unabsorbed compounds were removed from the surface with 70% ethanol by wiping several times. The treated skin was fixed with glutaraldehyde/paraformaldehyde. Dehydration was then performed with 30% sucrose. Cryo-embedded specimens were sectioned in 7 μm-thick slices on slide glass with a cryostat. The slides were then thawed and coated with emulsion solution. After 3 weeks of exposure in the light-proof boxes at 4°C, the emulsion-coated slide was developed for 3 minutes and stained using the H-E staining method (Stumpf et al., 2008).

RESULTS AND DISCUSSION

1. Conjugation of peptide with oleic acid

The KTTKS peptide was conjugated with oleic acid, as described in Fig. 1. Conjugation of the peptide with oleic acid was confirmed by mass spectroscopic analysis before and after conjugation. Prior to the conjugation reaction, a major peak at 564 corresponding to the KTTLS peptide was evident (Fig. 2A). After the reaction, the main peak appeared at 828 (Fig. 2B), indicating successful conjugation of the synthetic peptide with oleic acid. The product yield was 85.7% and was effective for peptide conjugation.

2. Nanosome preparation

We produced nanosomes by adding excess oleic acid to oleic acid-conjugated KTTKS, and then measured their sizes and shapes using ELS and cryo-TEM. The nanosomes were vortexed then homogenized using an Emulsiflex C-30, and their size and shape was confirmed using cryo-TEM. Fig. 3
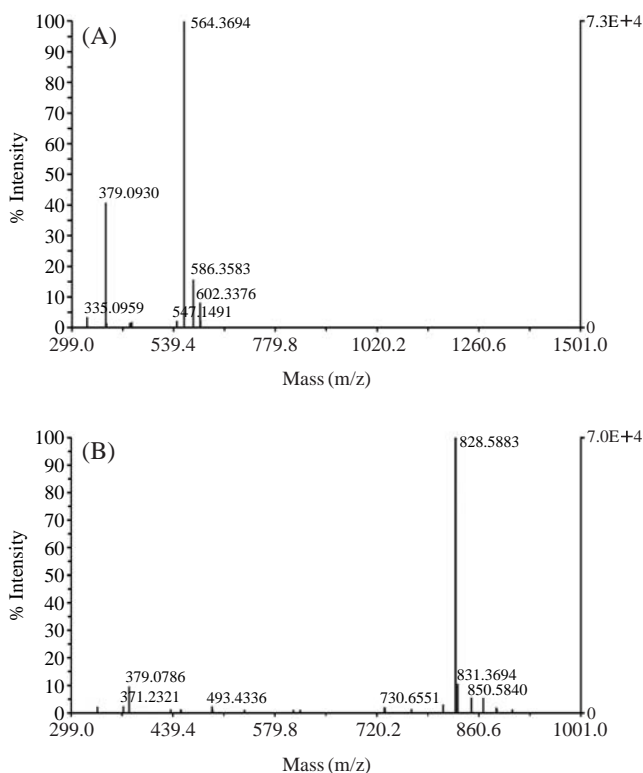
shows the typical structure of vesicles as liposomes after vortexing and homogenizing. The diagram (Fig. 3C) described the liposome structure which is made of mixture of additional oleic acid and conjugated peptide. After vortexing alone, vesicles were approximately 200 nm in size (Fig. 3A). After homogenization with Emulsiflex C-30, the vesicles were about 100 nm which was smaller than vortexing only method (Fig. 3B). Their size was a little difference but the shapes are similar between when using two different method. Fig. 4 shows nanosome sizes as measured by ELS. The overall size of nanosomes was stable as 150 nm for 24 weeks.
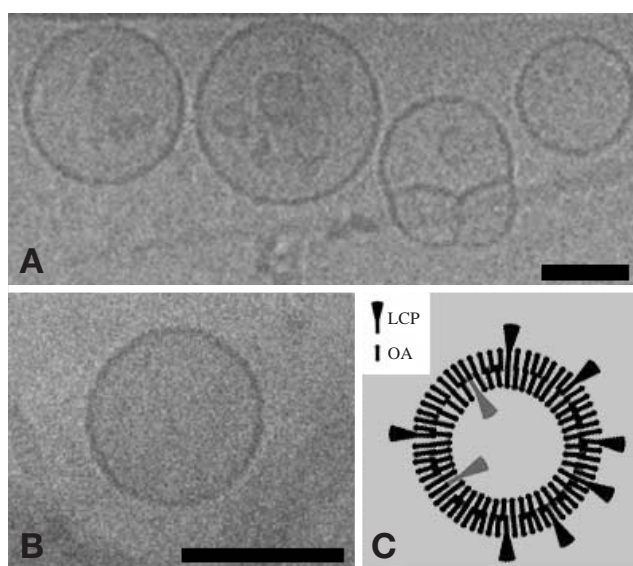

Fig. 3. Cryo-TEM of nanosome. A. nanosome produced by vortexing for 10 min. B. nanosome produced by Emulsiflex 10 cycles. Each bar is 100 nm. C. Diagram of oleic acid-conjugated peptide nanosome and oleic acid mixture. LCP; lipid (oleic acid) conjugated peptide, OA; oleic acid.

Fig. 2. Analysis of mass changes of synthetic peptide before and after conjugation with oleic acid using mass spectrometer. A. Mass spectrum of synthetic peptide before conjugation reaction. B. Mass spectrum of synthetic peptide after conjugation reaction.
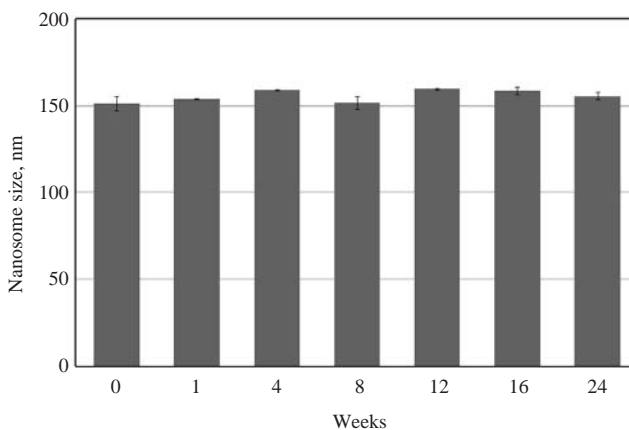

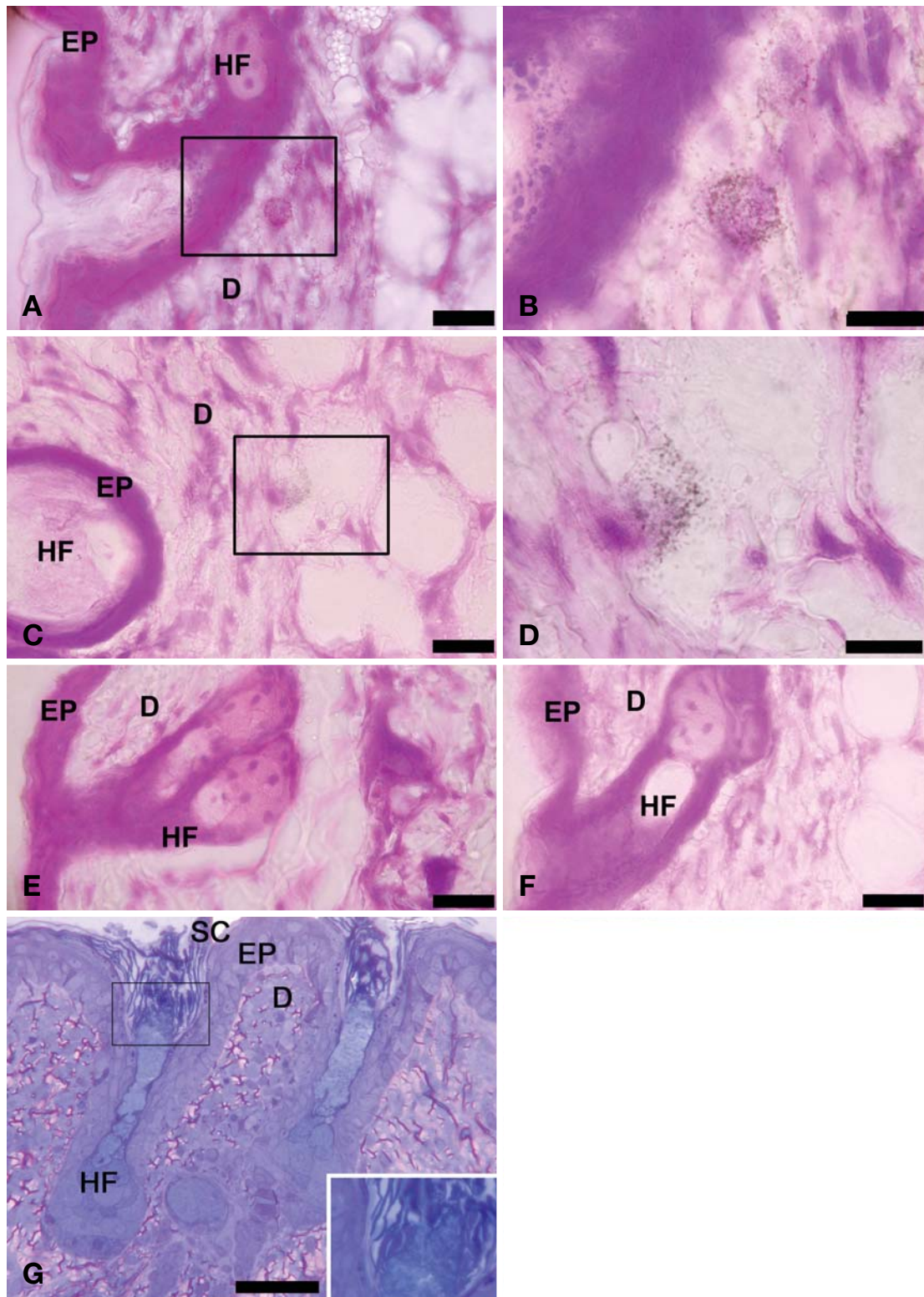**Fig. 4.** Graph of stability test of nanosomes.

Fig. 5. A, B, E Skin penetration patterns at 10 minutes after transdermal treatment. A. Penetration with nanosomes made of isotope-labeled peptide conjugated with oleic acid. B. Large magnified image marked area in A. E. Penetration with only isotope-labeled peptide in the absence of oleic acid conjugation. C, D, F. Skin penetration patterns at 45 minutes after transdermal treatment. C. Penetration with nanosomes made of isotope-labeled peptide conjugated with oleic acid. D. Large magnified image marked area in C. F. Penetration with only isotope-labeled peptide in the absence of oleic acid conjugation. G. Skin structure. Inbox shows the end of SC. EP; epidermis, D; dermis, HF; hair follicles, SC; stratum corneum. Bars: 20 μm in A, C, E, F, 10 μm in B, D and 50 μm in G.

KTTKS is composed of polar and charged amino acids. Conjugation of the large hydrophilic peptide with the hydrophobic oleic acid confers amphipathic properties to the molecule. Because of this amphipathic property, oleic acid-conjugated peptides are predicted to form micelles or liposomes in aqueous solutions with the hydrophobic region facing the interior of the molecule and the hydrophilic domain exposed at its surface as like the diagram (Fig. 3C). Liposomes are vesicles prepared from the same materials as the cellular membrane, and are commonly used biomaterials for drug or nutrient delivery. Certain properties of liposomes, including their potential for biodegradation and incorporation, make them attractive for use as a basic technology for cosmetology. The structure and size of liposomes used for transdermal delivery of cosmetics affect liposome stability and degree penetration (El Maghraby et al., 2000). Our results showed that oleic acid-conjugated KTTKS peptides could form vesicle-shaped nanosomes in the presence of excess oleic acid and they were remained stable for a long time. These properties make these peptides suitable for use in cosmeceuticals.

3. Penetration into skin

The nanosomes used in autoradiography experiments were prepared using the vortex method alone so as to avoid isotope contamination of the Emulsiflex C-30. Nanosome penetration was examined by autoradiography, and isotope-labeled materials appeared as dark grains in the skin tissue (Fig. 5). When 10 minutes after applying nanosomes prepared with ^{3}H-KTTKS-conjugated oleic acid to the skin, the nanosomes appeared in the dermis layer (Fig. 5A). In the large magnified image (Fig. 5B), dark grains were shown clearly. ^{3}H-KTTKS which was not conjugated with oleic acid was not detected within the skin tissue (Fig. 5E). After 45 min, nanosomes were also detected in the dermis layer (Fig. 5C, D), and the naked ^{3}H-KTTKS peptide without oleic acid conjugation was not detected (Fig. 5F). In the case of nanosome denoted small liposome in nanometer range (~200 nm), the penetration rate was related to both the size and the rigidity (Sundar et al., 2008). Our results showed the nanosome from oleic acid conjugated peptide appeared at early stage and that meams it could be penetrated fast. In detailed structure of skin layer with semi-thin section (Fig. 5G, inbox), there is end gap between appendage and stratum corneum. This property may account for the early penetration after plastering of nanosome. Imaging method such as CLSM and autoradiography can detect the target materials at early stage (Boderke et al., 1997). On the other hand diffusing methods

such as need enough amount of material and time for detection, so it is hard to detect the early stage. Our result of autoradiography was able to show early penetration of nanosome.

It has been reported that the KTTKS peptide has an effect on collagen regeneration. It is hard to apply as cosmeceuticals with KTTKS peptide itself, because this peptide is composed of polar and charged amino acids, and it has difficulty penetrating to the dermis through the lipidic environment of the stratum corneum. To circumvent this problem and improve skin penetration, we conjugated the KTTKS peptide with the fatty acid, oleic acid which is used frequently in pharmaceuticals (Smolinske, 1992). Nanosome Penetration was confirmed by autoradiography, which showed that the nanosomes were able to penetrate into certain layers of the skin. Our results indicated that nanosome from the oleic acid-conjugated peptide was able to penetrate into the dermis layer, whereas the peptide without oleic acid conjugation was unable to penetrate the skin. The polar and hydrophilic KTTKS peptide itself could not penetrate the lipid layer of stratum corneum, but amphipathic peptide by oleic acid conjugation was able to penetrate. From these results it is proposed that oleic acid conjugated nanosome is a good material for cosmeceuticals.

As described above, autoradiography was able to show precisely where penetration of the isotope-labeled materials occurred. Our autoradiography results showed that the nanosome containing isotope labeled peptides was able to penetrate through the stratum corneum. These results indicate that autoradiography is an appropriate method for clarifying the mechanism of penetration used by transdermal delivery systems.

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＜국문초록＞

Short 펩타이드는 약용화장품(cosmeceuticals)으로 가능성이 있지만, 펩타이드의 친수성 성질 때문에 피부를 통한 전달이 어렵다. 따라서 이러한 short 펩타이드를 약용화장품으로 이용하기 위해서는 피부를 통과할 수 있는 성질로 바꾸어야만 한다. 이 연구에서는 프로콜라겐 type I의 신호펩타이드로 작용할 수 있는 KTTKS의 5개 펩타이드에 양친매적인 특성을 부여하기 위해 올레산을 결합시켰고, 그 후 나노좀으로 제조하였다. 제조된 나노좀은 동결투과전자현미경법을 이용하여 크기가 100에서 200 nm 사이의 리포좀의 형태임이 확인되었으며, 제타전위측정장치(ELS)를 통해 24주 동안 안정적인 크기를 유지하고 있음이 확인되었다. 이렇게 제조된 나노좀을 피부에 바른 후 자가방사기록법(autoradiography)을 이용하여 피부에 침투 여부를 확인하였다. 이 5개 펩타이드의 친수성 성질로 인하여 펩타이드만 피부에 도말하였을 때는 피부에 침투되지 않았으나, 펩타이드와 올레산을 결합하여 나노좀으로 제조된 경우에는 피부의 진피층까지 잘 도달한 것을 확인하였다. 이 연구를 통해 자가방사법이 피부를 통한 효과물질의 전달을 검증할 수 있는 좋은 방법임을 보여줌과 동시에 피부에 효과를 나타내는 친수성펩타이드의 피부를 통한 전달에 올레산 결합을 이용한 나노좀 형태가 효과적인 것을 보여주었다.