Distribution of Polyphenols and a Surfactant Component in Skin during Aerosol OT Microemulsion-Enhanced Intradermal Delivery

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As for most other polyphenols, intradermal delivery of curcumin and resveratrol is limited; however, it was significantly improved by a microemulsion using Aerosol OT (Aerosol OT microemulsion) and Tween 80 (Tween 80 microemulsion) as surfactants. Aerosol OT microemulsion was more effective and the incorporation ratio of these polyphenols into skin by Aerosol OT microemulsion was five-fold or ten-fold that by Tween 80 microemulsion. To clarify the mechanism of the enhancement we examined the distribution of these polyphenols and the surfactant component, Aerosol OT, using excised guinea pig skin and Yucatan micropig (YMP) skin. During permeation, polyphenols distributed deep in the skin. In particular, a small molecule, resveratrol, was mainly present in the dermis in YMP skin. Aerosol OT also distributed deep in the skin. These findings suggest the possible involvement of the interaction of surfactant molecules with skin components in the enhanced delivery process of polyphenols. The distribution ratio between the dermis and epidermis of the polyphenols, including quercetin, in the presence of Aerosol OT microemulsion decreased with the increase of molecular weight in YMP skin, suggesting the possibility that distribution to the dermis is regulated by the molecular size.

Key words microemulsion; curcumin; resveratrol; Aerosol OT; skin; intradermal delivery

Microemulsions are thermodynamically stable and have been shown to have high solubilization capacity and to facilitate the skin permeation of both lipophilic and hydrophilic drugs.[1,2] Microemulsions consist of an aqueous phase, an organic phase, a surfactant and a co-surfactant component. Various surfactants, such as polyoxyethylene sorbitan monooleate (Tween 80) and lecithin, are used for the preparation of microemulsions, and short-chain alcohols are used as co-surfactants in many cases. Microemulsions have also been assessed as vehicles for the intradermal delivery of polyphenols to protect skin against ultraviolet (UV)-induced damage. For example, Vincentini *et al.* recently revealed the protective effect of the application of quercetin with a water-in-oil microemulsion, consisting of water, propylene glycol, Span 80 and Tween 80, against UVB-induced damage in hairless mouse skin.[3] We have also clarified the usefulness of another water-in-oil microemulsion system and oil-in-water microemulsion system, which consisted of 150 mM NaCl solution, isopropyl myristate (IPM), Tween 80 and ethanol, for the skin delivery of quercetin, genistein and chlorogenic acid to prevent against UV irradiation-induced erythema formation[4–6]; however, the mechanism of the enhancement by microemulsions is not clear. For that purpose it is necessary to clarify the distribution of microemulsion components, since it is still unknown.

In this study we examined the distribution of the polyphenols and a surfactant component in the skin by using excised guinea pig skin and Yucatan micropig (YMP) skin. We used di-2-ethylhexyl sodium sulfosuccinate (Aerosol OT, Fig. 1a) as a surfactant component in microemulsion, which has also been revealed as a useful surfactant component of microemulsions for topical drug delivery,[7,8] due to the ease of quantitative analysis. We examined the skin delivery of two polyphenols, curcumin and resveratrol, because like other polyphenols their intradermal and transdermal delivery are limited and the introduction of an enhancement system is expected for their topical application.[9,10] Curcumin (Fig. 1b) is the main colorant found in the rhizomes of *Curcuma longa* L., which exerts therapeutic effects on wound healing and skin tumors after topical application.[9,11] Resveratrol (*trans*-3,5,4′-trihydroxystilbene, Fig. 1c) is a naturally occurring polyphenolic phytoalexin synthesized by a wide variety of plant species, including grapes, berries and peanuts, in response to stress as a defense mechanism against fungal, viral, and bacterial functions, and damage from exposure to UV radiation.[10,12] In addition to these polyphenols we furthermore examined the skin distribution of quercetin (Fig. 1d) whose enhanced skin delivery we previously reported as a microemulsion using Tween 80 as a surfactant.[4] We investigated the relationship of the distribution ratio of these polyphenols between the dermis and epidermis with their molecular weight using YMP skin.

Experimental

Materials Aerosol OT, curcumin, quercetin, Tween 80, isopropyl palmitate (IPP) and isopropyl myristate (IPM) were obtained from Nacalai Tesque (Kyoto, Japan). Resveratrol and *trans*-ferulic acid were from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Ethanol and all other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dorsal skin was excised from Hartley strain male guinea pigs following the protocol approved by the Animal Experimentation Committee of Kobe Pharmaceutical University. Pentobarbital sodium was used for anesthesia. Subcutaneous fat and other extraneous tissues were trimmed before use. YMP skin set was purchased from Charles River Japan (Yokohama, Japan) and stored at −80°C until use. The fat and subdermal tissue were removed following the method of Fujii *et al.*[13]

Preparation of the Microemulsion Aerosol OT microemulsion was prepared using 150 mM NaCl solution as an aqueous phase and IPP as an oil phase, Aerosol OT as a surfactant, and ethanol as a co-surfactant at the weight ratio of

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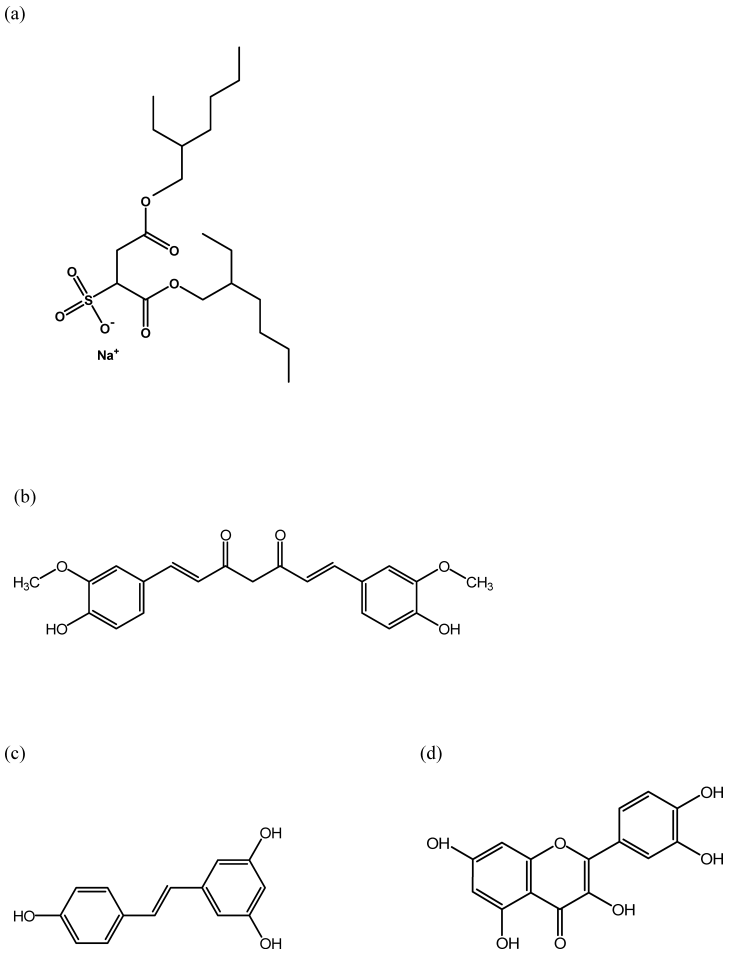

Fig. 1. Chemical Structures of Aerosol OT (a), Curcumin (b), Resveratrol (c) and Quercetin (d)

20.2 : 31.3 : 33.3 : 15.2 by modifying the method of Junyaprasert *et al.*, who used butanol instead of ethanol.[7] Ethanol also works as an aqueous phase ingredient in this microemulsion. Microemulsion using Tween 80 as a surfactant (named Tween 80 microemulsion in this study) was prepared using NaCl solution, IPM, Tween 80 and ethanol at the weight ratio of 7 : 33 : 30 : 30[4,5] by modifying the method of Lee *et al.*[14] The mean particle diameters of Aerosol OT microemulsion and Tween 80 microemulsion were 16.6±1.8 nm and 25.0±4.3 nm, which were measured using a particle analyzer FPAR-1000 (Otsuka Electronics Co., Hiratsuka, Japan). Each microemulsion was obtained by vortex mixing for a few minutes at 37°C. Curcumin or resveratrol was added to the pre-formed microemulsion at saturated concentration under solubilized conditions at 37°C.

Measurement of Intradermal Delivery *In vitro* study on the skin incorporation of curcumin and resveratrol was performed as described previously.[15] Guinea pig dorsal skin or YMP skin was mounted in a Franz-type diffusion cell with a water jacket (37°C). The available diffusion area was approximately 0.64 cm^2 and the receptor cell had a capacity of about 4.5 mL. After 2 h pretreatment of the skin with NaCl solution and washing both donor and receptor compartments, 1.0 mL vehicle (0.5 mL vehicle for guinea pig skin) containing each polyphenol was added to the donor compartment at saturated concentration under solubilized conditions, while phosphate-buffered saline (PBS (pH 7.4)) was added to the receptor compartment. After 20 h treatment (40 h treatment for YMP skin), at which time the amount of polyphenols penetrating the skin was close to the maximum level, the skin was removed from the cell, and the treated skin area was punched out and washed with ice-cold methanol. After drying at ambient temperature, the skin was weighed (0.065±0.018 g for guinea pig skin and about 0.145±0.036 g for YMP skin), minced and placed in 10 mL methanol, and then homogenized using a tissue homogenizer Polytron PT3100 (Kinematica, Lucerne, Switzerland) at 5000 rpm for 1 min.

The samples were then centrifuged and the supernatant layer was used for determination by HPLC using L-6000 equipped with L-4000 UV detector (Hitachi, Tokyo, Japan) for the polyphenols and PU-980 (JASCO, Tokyo, Japan) equipped with 996 photodiode array detector (Nihon Waters, Tokyo, Japan) for Aerosol OT. Separation was performed on a reversed-phase column (Mightysil RP-18 GP, 3.0 mm i.d., 150 mm) using a mobile phase consisting of methanol, water and phosphoric acid at the volume ratio of 120 : 80 : 1 for curcumin and 70 : 130 : 1 for resveratrol. The detection wavelength was 360 nm for curcumin, and 305 nm for resveratrol. Either quercetin or *trans*-ferulic acid was used for curcumin or resveratrol as the internal standard. For the analysis of Aerosol OT, a mobile phase consisting of methanol and water at the ratio of 80 : 20 was used. The detection wavelength was 210 nm and *trans*-ferulic acid was used as the internal standard. Analysis of quercetin by HPLC was carried out as described

previously.[4)]

The concentration of these polyphenols as well as Aerosol OT in the deeper skin layers of guinea pig skin was determined after freezing the skin rapidly with dry ice and methanol, transferring into a cryomicrotome, cutting the whole piece of skin into surface parallel sections and homogenizing as described above. From the concentration–depth profile obtained, the percentage of polyphenols penetrating to a certain depth of skin was calculated. The concentration of curcumin, resveratrol, quercetin and Aerosol OT in either the epidermis and dermis of YMP skin was measured after separation of the epidermis from dermis by a heat separation technique,[16)] and their distribution ratio between the dermis and epidermis was calculated.

Measurement of Solubility The solubility of curcumin and resveratrol was measured after incubation of an excess amount of these polyphenols in the microemulsions, 150 mM NaCl solution or IPM at 37°C for about 20 h. After brief centrifugation at 12000×***g*** for 1 min, the concentration of the supernatant was determined by HPLC as described above.

Measurement of Partition Coefficients Partition coefficients of curcumin and resveratrol between *n*-octanol and PBS (pH 7.4) were measured as described previously.[17)] PBS solution (3 mL) of polyphenols (0.1–3.0 mM) was mixed with 3 mL *n*-octanol in test tubes with glass stoppers. PBS and *n*-octanol solutions were pre-saturated with either *n*-octanol or PBS and deoxygenized with a nitrogen stream. The test tubes were set at 37°C for 18 h in a shaking water bath. After shaking, incubation continued for another hour. The concentration of polyphenols in the PBS phase and *n*-octanol phase was determined by HPLC as described above.

Statistical Analysis Data were analyzed using the Kruskal–Wallis test. Individual differences between medians were examined using Dunn's multiple comparison test.

Results

Enhanced Skin Delivery of Curcumin and Resveratrol by Microemulsions We first examined the effects of microemulsions on the skin delivery of curcumin and resveratrol, using guinea pig skin and YMP skin as model skin. They have been utilized for skin permeability studies and have been clarified to be good models to predict human skin permeability, although solute permeability in guinea pig skin is higher than in human skin, and that in YMP skin is lower.[13,18,19)]

Logarithm values of the partition coefficients between *n*-octanol and PBS for curcumin and resveratrol were 3.68 and 2.97, respectively; therefore, these polyphenols are relatively hydrophobic. As shown in Fig. 2 for the results using guinea pig skin, accumulation of these polyphenols from control vehicle IPM was very limited, as we previously reported for quercetin and isoflavones.[4,5)] In particular, accumulation of curcumin was less than that of resveratrol. As shown in the same figure, microemulsions markedly increased the skin accumulation of these polyphenols. Compared with the accumulation from IPM, about a 70-fold increase was observed for curcumin and about a 14-fold increase was observed for resveratrol in the accumulation from Aerosol OT microemulsion. The increases by Aerosol OT microemulsion were larger than those by Tween 80 microemulsion for both polyphenols, although the solubilities of these polyphenols were smaller in Aerosol OT microemulsion, as described in Table 1; therefore,

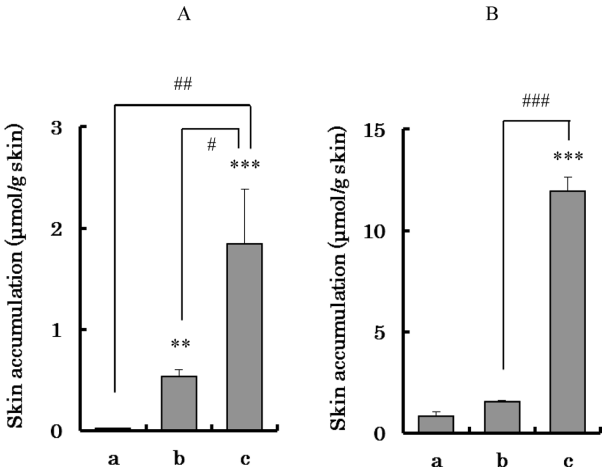

Fig. 2. Skin Accumulation of Curcumin (A) and Resveratrol (B) in Guinea Pig Skin When Applied in IPM (a), Tween 80 Microemulsion (b) or Aerosol OT Microemulsion (c) at Concentration Shown in Table 1

Data are the means±S.D. of four experiments. $^{**}p<0.01$, $^{***}p<0.001$, significantly different from the values in IPM. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, significantly different among the data.

Table 1. Effects of Microemulsions on Solubility of Curcumin and Resveratrol

Vehicle	Solubility (mM)	
	Curcumin	Resveratrol
150 mM NaCl solution	0.026±0.009	0.259±0.005
IPM	0.52±0.01	1.19±0.04
Tween 80 microemulsion	49.9±4.2***[a)]	149.1±7.1***[a,b)]
Aerosol OT microemulsion	5.29±1.02***	56.6±3.7***

Data are the means±S.D. of four experiments. $^{***}p<0.001$, significantly different from the values in NaCl solution and IPM. *a*) $p<0.001$, significantly different from the values in Aerosol OT microemulsion. *b*) Solubility was not measured because microemulsion broke down at higher concentrations.

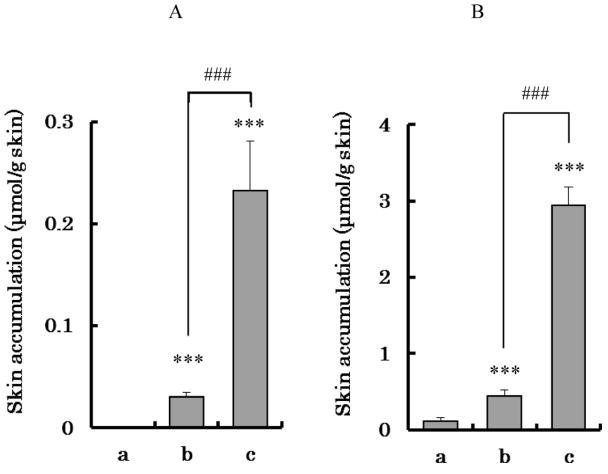

Fig. 3. Skin Accumulation of Curcumin (A) and Resveratrol (B) in YMP Skin When Applied in IPM (a), Tween 80 Microemulsion (b) or Aerosol OT Microemulsion (c) at Concentration Shown in Table 1

Data are the means±S.D. of four experiments. $^{***}p<0.001$, significantly different from the values in IPM. $^{\#\#\#}p<0.001$, significantly different among the data. Curcumin was not detected when applied with IPM.

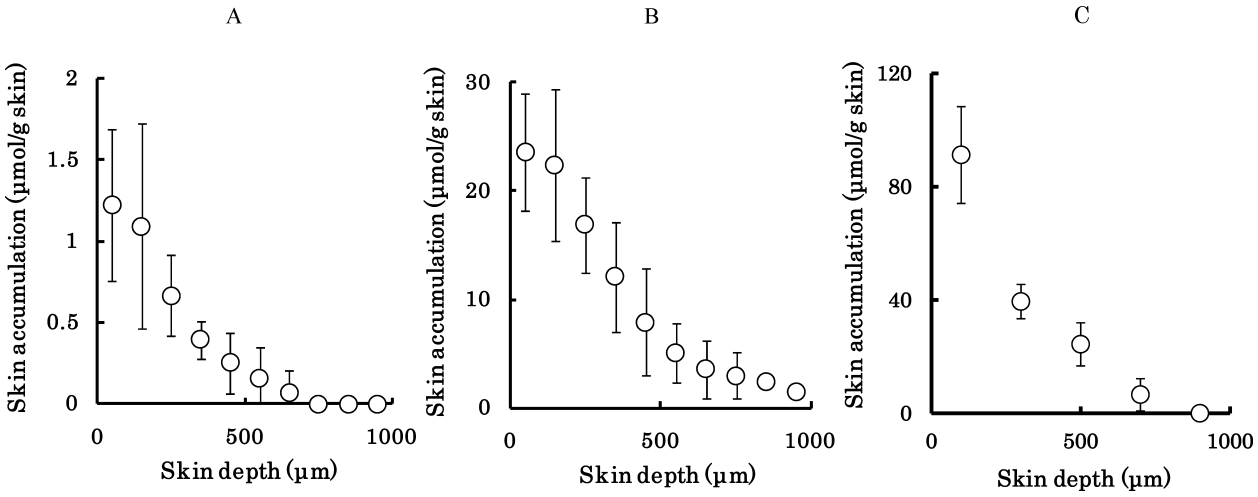

Fig. 4. Concentration–Depth Profile of Curcumin (A), Resveratrol (B) and Aerosol OT (C) in Guinea Pig Skin When Each Polyphenol Was Applied with Aerosol OT Microemulsion at Saturated Concentration (5.29 mM Curcumin, 56.6 mM Resveratrol, Respectively)

Data are the means±S.D. of three experiments.

the efficiency of skin incorporation was much higher in Aerosol OT microemulsion. About 1.7% curcumin and about 2.2% resveratrol added to the donor compartments were incorporated into skin by Aerosol OT microemulsion, whereas only about 0.21% curcumin and about 0.15% resveratrol were incorporated into skin by Tween 80 microemulsion.

As shown in Fig. 3, enhanced skin delivery by microemulsions, especially by Aerosol OT microemulsion, was also confirmed in YMP skin, which is hairless and has been suggested to be a good model to predict human skin permeability,[19] as well as showing physiological similarity to human skin.[20]

Distribution of Polyphenols and Aerosol OT in Skin To investigate the mechanism of the skin delivery of these polyphenols by Aerosol OT microemulsion, the distribution of polyphenols and Aerosol OT at various depths of guinea pig skin was compared. The concentration–depth profiles of these polyphenols and Aerosol OT were observed by cutting the skin into surface parallel sections using a cryomicrotome. We selected this method to see the skin distribution of these compounds, because relatively high permeability of guinea pig skin was suitable for this analysis. Like quercetin, whose increasing effect on solubility and enhanced skin distribution we previously reported for Tween 80 microemulsion,[4] the concentration of these polyphenols gradually decreased throughout the skin as shown in Fig. 4, and their distribution in deep skin layers was confirmed. In particular, resveratrol was found even in the bottom layer of the skin. Aerosol OT was also distributed in the deep skin layer. Overall, about 0.93% of Aerosol OT in the vehicle was incorporated into skin, and its concentration–depth profiles resembled those of curcumin.

We also examined the distribution of these polyphenols and Aerosol OT in YMP skin by separating the epidermis and dermis after their absorption from the donor compartment of Franz-type diffusion cells and observing their accumulation in each skin sample in order to confirm the penetration of these compounds to the dermis in the hairless skin. We selected this method for YMP skin to see the skin distribution of polyphenols and Aerosol OT because quantitative analysis in thin skin slice samples was difficult due to the much slower penetration of these compounds in YMP skin compared with the penetration in guinea pig skin. Furthermore, we selected this method to try to clarify the distribution ratio of these compounds between the dermis and epidermis of hairless skin, because the physico-chemical property of the solutes which regulate the distribution from the epidermis to the dermis is not clear. To see the difference of the skin distribution of polyphenols clearly, total accumulated amounts in the epidermis and dermis are shown in Fig. 5. From the findings, the penetration of the polyphenols and Aerosol OT into the dermis was confirmed in hairless skin. As shown in Fig. 5A, the ratio of the accumulated amount in the epidermis and dermis was about 2 : 1 for curcumin. On the other hand, the ratio of that for a smaller molecule, resveratrol, was about 1 : 3, as shown in Fig. 5B. Therefore, most resveratrol molecules were present in the dermis. We also examined the skin distribution of another polyphenol, quercetin. Its logarithm value of the partition coefficients between *n*-octanol and PBS was 2.74 (less than those of curcumin and resveratrol).[4] Quercetin accumulated in the epidermis and dermis to a similar extent as shown in Fig. 5C. A surfactant component, Aerosol OT, was also found in both the epidermis and dermis. Its accumulated amount in the epidermis was double that in the dermis, as shown in Fig. 5D.

We calculated the distribution ratio of the polyphenols, including quercetin, between the dermis and the epidermis and examined the relationship of their logarithm values with their molecular weights. As shown in Fig. 6, we found a tendency for the distribution ratio to decrease with the increase of the molecular weight.

Discussion

Microemulsions offer several advantages for pharmaceutical use, such as long-term stability, high solubilization capacity for both hydrophilic and lipophilic drugs, and improved dermal and transdermal drug delivery.[21] We have revealed that microemulsions using Tween 80 are useful for the skin delivery of polyphenols whose solubility in aqueous and non-aqueous vehicles is limited, because they markedly improved the skin incorporation of polyphenols as well as their solubility.[4,5] The findings obtained in this study revealed that using

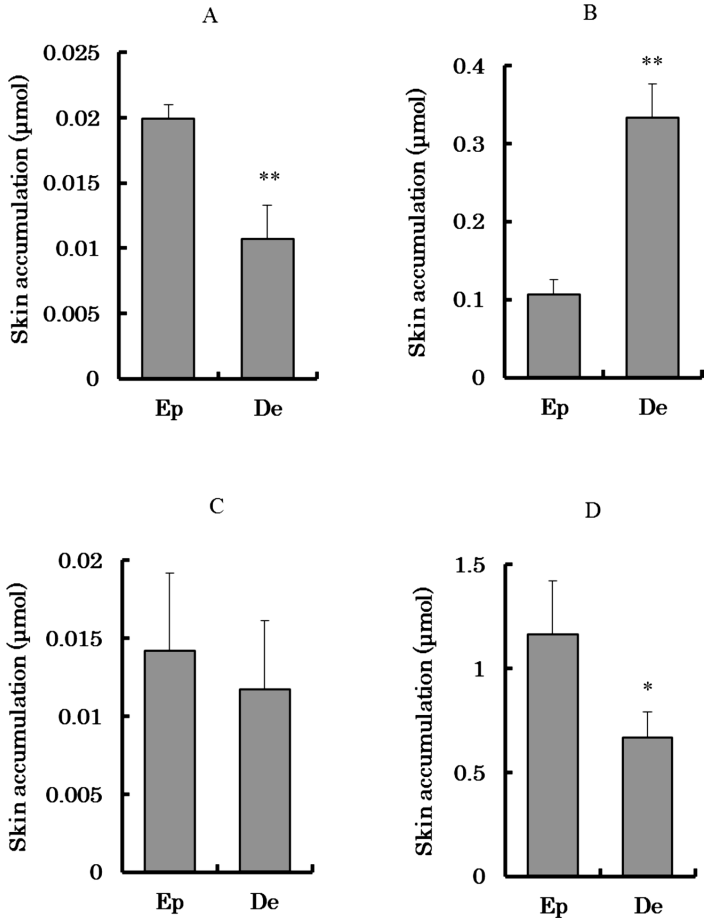

Fig. 5. Total Accumulated Amount of Curcumin (A), Resveratrol (B), Quercetin (C) and Aerosol OT (D) in Epidermis (Ep) and Dermis (De) of YMP Skin When Each Polyphenol Was Applied with Aerosol OT Microemulsion at Saturated Concentration

Data are the means±S.D. of three experiments. Concentration of quercetin was 4.11 mM. *$p<0.05$, **$p<0.01$, significantly different from the value in the epidermis.

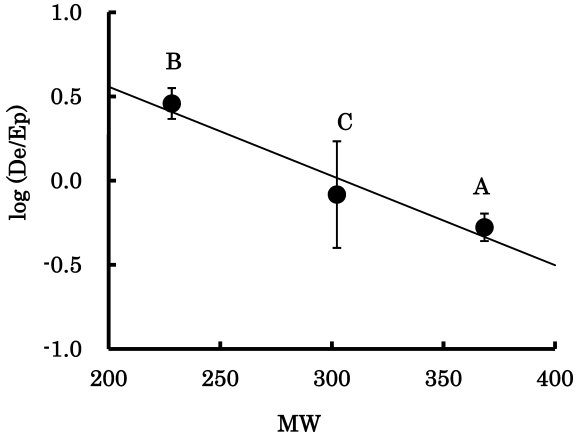

Fig. 6. Relationship of the Distribution Ratio between Dermis (De) and Epidermis (Ep) of the Polyphenols with Their Molecular Weight

(A) Curcumin; (B) resveratrol; (C) quercetin. Data are the means±S.D. of three experiments.

Aerosol OT microemulsion, which consisted of 150 mM NaCl solution, IPP, Aerosol OT and ethanol, efficient skin accumulation was found for the relatively hydrophobic polyphenols curcumin and resveratrol, although the solubility of these polyphenols was lower than that in Tween 80 microemulsion. Aerosol OT microemulsion is therefore of benefit for the skin delivery of these polyphenols.

Although various mechanisms have been postulated on the enhancement of skin permeation by microemulsions, the precise mechanism is still unknown. To clarify this, it is necessary to reveal the distribution of microemulsion components, especially that of a surfactant component. In this study we have revealed that a surfactant component, Aerosol OT, penetrated into deep skin layers. This finding suggests the possible involvement of the interaction of surfactant molecules with skin components in the enhanced delivery process of polyphenols. In particular, the major presence of surfactant molecules in the epidermis suggests the possibility that microemulsions mix their components with the lipid lamella of the stratum corneum and enhance the permeation of polyphenols through the stratum corneum. Interaction of a cosurfactant, ethanol, with skin components may also be involved in the enhanced delivery by microemulsions, as described previously for Tween 80 microemulsion.[4]

In this study it was also clarified that all the polyphenols tested penetrated the demis in YMP skin. In particular, a smaller molecule distributed in the dermis at a higher ratio. We used two kinds of mammalian skin as model skin for human skin, which are different in structure and solute permeability.[13,18,19] Using the value of 120 μm[22] as the thickness of the epidermis, it was estimated from the results in Fig. 4 that 62.5% of curcumin present in guinea pig skin distributed in the dermis, whereas a higher 71.5% of a smaller molecule,

resveratrol, distributed in the dermis. According to the same estimation, only 6.2% of curcumin present in guinea pig skin distributed to depths of more than 500 μm from the skin surface, whereas 16.2% of resveratrol distributed in the same skin area. Therefore, the tendency for a smaller molecule to penetrate a deeper skin layer at a higher ratio was the same for both skins; however, the difference of skin distribution among polyphenols seems to be much less in guinea pig skin. The presence of hair follicles in guinea pig skin and the relatively low barrier function of its stratum corneum may affect the distribution.

The distribution ratio between the dermis and epidermis of the polyphenols, including quercetin, in the presence of Aerosol OT microemulsion decreased with the increase of molecular weight in YMP skin. This finding suggests that the distribution of polyphenols from the epidermis to the dermis depends on their molecular size. It has been reported that diffusion in the dermis is regulated by the molecular size of the solutes.[23] Their molecular size-dependent diffusion process may also regulate the distribution of polyphenols in the dermis at steady state, although the reason is not clear. Dermis is not a homogeneous tissue. It consists of several kinds of cells such as fibroblasts and matrix components such as collagen fibers, which are a major component of the dermis; therefore, the regulation of distribution in and permeation of the dermis seems to be complicated. Further study of many solutes in the presence and absence of an enhancement system is necessary to reveal the correlation between the skin distribution of solutes and their molecular weights, and furthermore to reveal the regulation mechanism of solute distribution between the epidermis and the dermis.

Conclusion

Aerosol OT microemulsion, consisting of 150 mM NaCl solution, IPP, Aerosol OT and ethanol, significantly improved the intradermal delivery of curcumin and resveratrol at high efficiency compared with the Tween 80 microemulsion. Observation of the distribution in guinea pig skin and YMP skin revealed that a surfactant molecule, Aerosol OT, as well as curcumin and resveratrol, penetrated deep in the skin. The findings suggest the possible involvement of the interaction of surfactant molecules with skin components in the enhanced delivery process of polyphenols. Among polyphenols, including quercetin, a smaller molecule, resveratrol, distributed in deeper skin layers. The distribution ratio between the dermis and epidermis of the polyphenols decreased with the increase of molecular weight in YMP skin, suggesting the possibility that the distribution is regulated by the molecular size.

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