

Effects of methyl paraben on skin keratinocytes

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ABSTRACT: Some ingredients of dermatological formulations result in skin irritation and allergy. In particular, preservatives have been reported extensively as a cause of allergic contact dermatitis. The study focused on parabens which have been used extensively as antimicrobial preservatives in foods, drugs and cosmetics. The aim of this study was to clarify the effects of the daily use of methyl paraben (MP) on human skin.

The concentrations of MP in the stratum corneum (SC) of the human forearm were measured using the cup method and GC-MS after daily applications of MP containing formulations. The study also investigated the effects of long-term exposure to MP on keratinocytes *in vitro*. Normal human keratinocytes and the skin equivalents were cultured in the medium containing MP. The following changes were analysed: proliferating ability, apoptotic cells, morphological changes, mRNA and protein expressions.

After 1 month of daily applications of MP containing formulations, MP remained unmetabolized and persisted slightly in the SC. MP decreased the proliferating ability of keratinocytes and changed the cell morphology. MP also decreased the expressions of hyaluronan synthase 1 and 2 mRNAs and type IV collagen. In contrast, it increased the expressions of involucrin and HSP27. Furthermore, MP influenced the epidermal differentiation of the skin equivalent.

These results suggest that MP exposure through application of dermatological formulations results in MP persistence and accumulation in the SC, and that MP might influence the aging and differentiation of keratinocytes. Copyright © 2006 John Wiley & Sons, Ltd.

KEY WORDS: parabens; methyl paraben; keratinocytes; aging; differentiation

Introduction

The stratum corneum (SC), formed by the proliferation and differentiation of epidermal keratinocytes, is an important tissue as a protector against water loss, and as a barrier for environmental stresses, for example, pollution, dry atmosphere and UV radiation. These stresses lead to dermatological disorders. Some ingredients of dermatological formulation result in skin irritation and allergy and can be also considered as stressors of the skin. Among these ingredients, antimicrobial preservatives have been reported extensively as a cause of allergic contact dermatitis (Wilkinson *et al.*, 2002; Sasseville, 2004). Although cosmetics not containing preservatives have appeared on the market, expanding the concept of 'sensitive skin' and the concentration of preservatives has been decreased by the manufacturers, almost all cosmetics contain some preservatives, such as parabens.

Parabens are esters of 4-hydroxybenzoic acid. The six commonly used forms of paraben are methyl paraben

(MP), ethyl paraben, *p*-propyl paraben, isobutyl paraben, *n*-butyl paraben and benzyl paraben. They are used extensively as antimicrobial preservatives in food, cosmetics and pharmaceutical products because of their broad spectrum of activity, inertness and low cost. Parabens are used in large quantities daily. Parabens are generally considered as safe preservatives, because parabens are rapidly absorbed and metabolized into *p*-hydroxybenzoic acid (PHBA), which is less toxic than parabens.

However, parabens have been reported recently to possess an estrogenic potential (Lemini *et al.*, 1997; Lemini *et al.*, 2003; Lemini *et al.*, 2004; Darbre *et al.*, 2004), this has generated renewed interest in the influence of parabens on human skin.

Under these circumstances, this study investigated the effects of MP (the most common of the six forms of parabens), which was absorbed percutaneously through daily use of MP containing formulations on the skin, especially SC and epidermal keratinocyte.

MP was studied from the viewpoint of its absorption and accumulation via daily topical application. This paper reports the results of the evaluation studies conducted to determine whether MP in dermatological formulations persists in the human skin and the effect of long-term exposure to low MP concentrations as a stressor on skin keratinocytes *in vitro*.

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Materials and Methods

Reagent

Methyl paraben was purchased from Midori Kagaku Co., Ltd (Tokyo, Japan).

Measurements of MP Concentrations in Human Forearm Skin

The test population comprised healthy Japanese adults. Informed consent was obtained from each of the subjects before they participated in the study.

The formulations used in the study were composed of commonly used ingredients such as water, butylene glycol, propylene glycol, glycerin (only emulsion) and squalane (only emulsion), ethanol was excluded from the formulations.

To examine the concentration of MP in the SC after a single application of a MP containing formulation, emulsions (0.15 g each) containing 0.15%, 0.25% and 0.5% (w/v) of MP were applied to the forearm area of three volunteers (two men and a woman) (area 42 cm²). At 1, 2, 5 and 12 h after the application, the MP in the SC was extracted using the cup method with 0.5 ml ethanol as a solvent for 5 min after wiping off the emulsion on the skin's surface with wet cotton. The solvent was applied to the skin of the forearm using a glass cylinder with a cross-sectional area of 3.1 cm². MP concentrations were determined using a GC-MS system or a HPLC system described below.

To examine the concentration of MP in the SC repeated application of MP containing formulations, 12 Japanese volunteers (1 man and 11 women) aged in their 20s and in their 50s were the subjects of this test. Each subject applied test formulations to both the forearms twice a day for 1 month. Six subjects applied only a lotion, while the other six subjects applied the lotion and an emulsion. The subjects stopped the use of any other formulations on the test area during the test period and bathed as usual. On the morning of the measurement, the test formulations were not used. The MP was extracted using the cup method described above, and MP concentrations were determined using a GC-MS described below.

GC-MS Analysis

The extracted solution (4 µl) was directly introduced into a QP-5050 GC-MS-SIM (m/z = 121,152) system (Shimadzu, Kyoto, Japan) by the splitless injection method, employing electron impact ionization at 70 eV. A DB-1 capillary column (0.25 mm i.d. × 30 m; J&W Scientific, CA) was used for the GC separation. The initial temperature of the column was 80 °C. After 1 min,

the temperature was increased to 250 °C at a rate of 15 °C min⁻¹, then to 310 °C at a rate of 20 °C min⁻¹, and finally maintained at 310 °C for 5 min. Helium gas was used as the carrier at a flow rate 1.5 ml min⁻¹. In this system, minimum 0.02 pmol cm⁻² of MP was detected.

HPLC Analysis

The HPLC system (Shimadzu model LC-10A system, Shimadzu Co., Kyoto, Japan) was equipped with a spectrophotometric detector (SPD-M10AVP) that was used to detect the high MP concentration at 1, 2 and 5 h after a single application, because a minimum detectable concentration of MP was 5 pmol cm⁻². Using a methanol–water (60:40) mixture as the mobile phase and a flow rate of 0.8 ml min⁻¹, the HPLC analysis was performed on a reversed-phase column (150 mm × 4.6 mm i.d.; Tsk-Gel 80Ts, Tosco Inc., Tokyo, Japan), with UV detection at 254 nm.

In Vitro Epidermal Diffusion Studies

Skin samples, excised from a Yucatan micropig (female, 5 months of age), were purchased from Charles River Japan, Inc. (Yokohama, Japan) in the frozen state at –80 °C.

Modified Franz-type diffusion cells with a surface area of 0.95 cm² for transport were used for the studies. The receptor compartment was filled with phosphate-buffered saline (PBS, ca. 4 ml, pH 7.0), which was maintained at 37 °C, and stirred with a magnetic stirrer at 400 rpm. Prior to the experiment, the skin sample was thawed at room temperature for approximately 30 min. The remnant fatty layers were excised carefully with scissors, and the skin sample was then cut into sections measuring ca. 2 cm × 2 cm. After the skin sections were placed into the apparatus, 10 µl of 0.1% (w/w) MP aqueous solution was spread on the SC side of the skin. Replication was $n = 5$ and two additional cells dosed with the vehicle alone served as the controls.

At 15, 60 and 120 min after unocclusive application of the solution, the skin samples were removed from the diffusion cell and carefully wiped with paper moistened with a methanol–water (50:50) mixture. The diffusion area of the SC was removed by five strippings with an adhesive tape (3M-Scotch). Ten milliliters of the methanol–water (50:50) mixture was added to the tape strips, left overnight, shaken for 1 min and then filtered. The epidermis, consisting of the SC and the viable epidermis, was separated from the dermis by a heat separation technique. The separated tissues were weighed, and then minced with scissors and homogenized with 4 ml methanol–water (50:50) mixture by using a Polytron PT-MR3000 homogenizer (Kinematika AG,

Switzerland). After centrifugation, the supernatant was used after filtering it through a 0.45 µm membrane filter (Advantec TOYO, Tokyo, Japan). MP concentrations were determined as mentioned earlier. Using a methanol–water (50:50) mixture as the mobile phase and a flow rate of 0.7 ml min⁻¹, the HPLC analysis was performed on a reversed-phase column (150 mm × 4.6 mm i.d.; Tsk-Gel 80Ts, Toso Inc., Tokyo, Japan) with UV detection at 254 nm.

Cell Culture and MP Treatment

Normal human epidermal keratinocytes-neonatal (NHEKs) were purchased from Cambrex (San Diego, California) and cultured in the supplied EpiLife-KG2 medium (Cascade Biologics, Portland, Oregon) containing 0.001% and 0.003% MP. When the cells reached subconfluence, they were subcultured in a medium containing MP. The cells cultured in a medium not containing MP were used as the controls. A Coulter counter was used to determine viable cell numbers. This count allowed the determination of the number of population doublings. Population doublings were calculated at each passage: (log₁₀ cells harvested-log₁₀ cells plated)/log₁₀ 2.

Detection of Apoptotic Cells

Apoptosis in the cells, which were cultured with MP (0.001%, 0.003%) or without MP for 4, 8, 16 and 32 days, was detected by annexin V binding using a commercially available kit (Vybrant Apoptosis Assay Kit #2, Molecular Probes, Netherlands). Adherent NHEKs, which were cultured in two-well chamber slides (Lab-Tek® Chamber Slide™ System; Nalge Nunc International, USA), were washed with PBS and exposed to annexin V binding buffer. Alexa Fluor® 488-conjugated annexin V (1 µg ml⁻¹) was added to the wells, and the cells were incubated at room temperature for 15 min. After washing with binding buffer, the cells were mounted on coverslips, and the numbers of viable (not stained), early apoptotic (annexin V stained, green fluorescence) and necrotic cells (propidium iodide stained, red fluorescence) were quantified using fluorescence microscopy. The total numbers of cells in five fields of view were counted and categorized in duplicate or triplicate experiments.

Fluorescence Staining of F-actin

The cells were fluorescently stained at 0, 16 and 32 days. The NHEKs were subcultured in chamber slides for 4 days. The cells were fixed in 3.7% formaldehyde in PBS for 10 min and were permeabilized with 0.1% Triton

X-100 in PBS for 5 min at room temperature. The cells were preincubated with 1% bovine serum albumin in PBS for 30 min at room temperature and then incubated with 80 nM Alexa Fluor® 568-conjugated phalloidin (Molecular Probes) and 1 µg ml⁻¹ DAPI (Sigma) in PBS for 20 min at 37 °C. The cells were observed under a fluorescence microscope.

Real-time RT-PCR

Using the RNeasy Mini Kit (Qiagen), total RNA was isolated from the NHEKs cultured for 16 days in the medium with or without MP. The total RNA was reverse-transcribed to cDNA using the First-Strand cDNA Synthesis Kit (Amersham Biosciences). The real-time PCR assay was performed using the QuantiTect SYBR Green PCR Kit (Qiagen). The relative expression values were normalized to the expression value of GAPDH as an internal control. The primers were as follows: for HAS1, sense primer 5'-TCT GTG ACT CGG ACA CAA GG-3' and antisense primer 5'-AGT ATC GCA GGC TGC TTA GG -3'; for HAS2, sense primer 5'-TTG GCA TCA CAC CTC ATC AT-3' and antisense primer 5'-GAT GCA AAG GGC AAC TGT TT-3'; for HAS3, sense primer 5'-GAG TCA GTG GTC ACG GGT TT-3' and antisense primer 5'-CGT CAG CAG GAA GAG GAG AA-3'; for GAPDH, sense primer 5'-CGA CCA CTT TGT CAA GCT CA-3' and antisense primer 5'-TTC CTC TTG TGC TCT TGC TG-3'.

Western Blots

Proteins were extracted from the NHEKs cultured for 16 days in the medium with or without MP by using a lysis buffer (20 mM Tris-HCl (pH 7.5), 0.4% NP-40, 150 mM NaCl). Equal amounts of protein were separated by SDS-PAGE that was performed on a 12.5% gel for involucrin, or on 5%–20% gradient gels for type IV collagen and HSP27 under reducing conditions. The separated proteins were stained with a silver staining kit (Wako, Osaka, Japan). For immunoblotting analyses, the proteins resolved by SDS-PAGE were transferred to a PVDF membrane, and detection was performed using the ECLplus detection reagents (Amersham Biosciences). The following primary antibodies were used for immunoblotting: anti-type IV collagen (1:500; Chemicon), anti-HSP27 (1:5000; StressGen) and anti-involucrin (1:5000; Sigma).

Skin Equivalent Culture and MP Treatment

Cultured skin equivalents (Testskin™; airlifted for 1 week) were purchased from Toyobo Co., Ltd. (Osaka,

Japan). The skin equivalents were subsequently maintained at the air–liquid interface for up to 14 days (after purchase, the equivalents were maintained for an additional 7 days). The culture medium was supplemented with 0% and 0.003% MP, and changed every 2 or 3 days.

The skin equivalents were fixed in the insert using 10% neutral buffered formalin and embedded in paraffin wax. The sections were stained with Mayor's hematoxylin–eosin for histological examination.

The parts of skin equivalents were homogenized in lysis buffer (20 mM Tris-HCl pH 7.5, 0.4% NP-40, 150 mM NaCl) with a digital homogenizer and centrifuged at 17 000 rpm for 5 min, and the pellets were discarded. The supernatants were used for determination of HSP27 by Western blotting mentioned earlier.

Results

MP Concentration in the SC after Single MP Application

To evaluate the percutaneous MP absorption, the MP concentration in the SC was measured using the cup method after a single application of the emulsions. MP concentration of the untreated area was very low (described as 0 h in Fig. 1). At 1 h after the application of the test formulation containing 0.15% MP, the MP concentration in the SC was approximately $6320 \text{ pmol cm}^{-2}$. This was approximately 18% of the application quantity of MP, and increased in a dose-dependent manner. However, it decreased with passage of time. At 12 h after the application of the test formulation containing 0.15% MP, the MP concentration in the SC was approximately

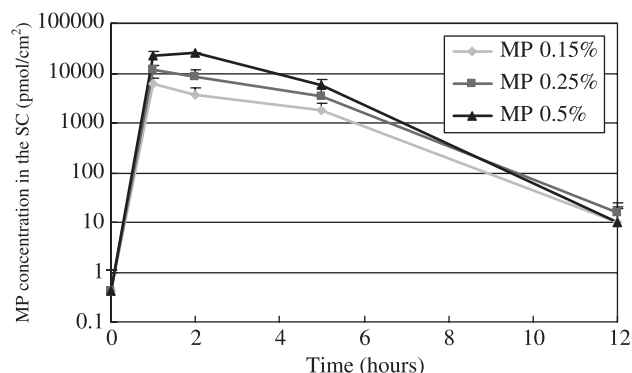


Figure 1. MP concentration in the SC after a single application of MP. Each value (mean $\pm$ SD) represents the average MP concentration (pmol cm^{-2}) in the SC ($n = 3$). At 0 h, MP concentration of one subject was below the minimum detectable concentration by GC-MS. The minimum detectable concentration, $0.02 \text{ pmol cm}^{-2}$, was used for the calculation

10 pmol cm^{-2} , or about 0.028% of the application quantity of MP (Fig. 1).

MP Concentration in the SC after Repeated MP Application

To determine the MP concentration in the skin of people using dermatological formulations containing MP, the MP concentration was measured in the SC of human skin treated with test formulations twice a day for 1 month. In considering the daily and common use of formulations, two groups using lotion only or lotion and emulsion were designed. The MP concentration remaining in the SC increased with time throughout the experimental period, with both groups noting the same tendency. The treatment with the lotion and emulsion containing MP resulted in higher MP concentrations in the SC than that produced by the treatment with only the lotion. However, after the subjects stopped using the test formulations, the MP concentrations decreased to the baseline level (Fig. 2).

Epidermal Diffusion of MP after a Single Application

To estimate the MP concentration in the epidermis, the MP concentration was measured in the dorsal skin of the Yucatan micropig after a single application of MP.

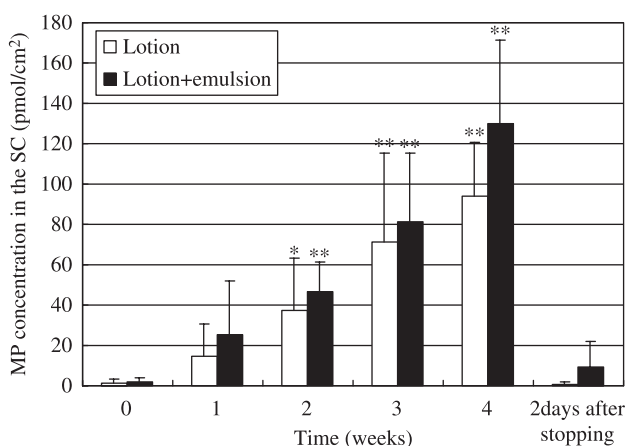


Figure 2. MP concentration in the SC after repeated application of MP. Each value (mean $\pm$ SD) represents the average MP concentration (pmol cm^{-2}) in the SC ($n = 6$). MP was not detected in 5 of 6 subjects who applied lotion, and 3 of 6 subjects who applied both lotion and emulsion at week 0. The minimum detectable concentration, $0.02 \text{ pmol cm}^{-2}$, was used for the calculation. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$) from week 0

Table 1. MP concentrations ($\mu\text{g g}^{-1}$ tissue weight) in the micropig skin after a single application of MP

		Time after MP application		
		15 min	60 min	120 min
MP	Stratum corneum	976 $\pm$ 104	1651 $\pm$ 276	2046 $\pm$ 193
	Epidermis	9.2 $\pm$ 0.6	19.3 $\pm$ 1.5	15.0 $\pm$ 3.4
	Dermis	2.3 $\pm$ 0.2	3.2 $\pm$ 0.7	3.4 $\pm$ 0.6
PHBA	Stratum corneum	ND	129 $\pm$ 53	319 $\pm$ 64
	Epidermis	8.7 $\pm$ 1.3	15.2 $\pm$ 1.4	22.2 $\pm$ 1.9
	Dermis	2.0 $\pm$ 0.2	9.9 $\pm$ 0.3	15.3 $\pm$ 2.1

Each value (mean $\pm$ SE) represents the MP concentration ($n = 4$).

Table 1 shows the MP concentrations in the SC, epidermis and dermis. One hour after the application, the MP concentration in the epidermis was $19.3 \pm 1.5 \mu\text{g g}^{-1}$ tissue. It accounted for approximately 0.002% of the tissue weight in percent figures. This result was used to investigate the effects of MP on keratinocytes.

Proliferating Ability of NHEKs

The effects of long-term MP exposure on NHEKs were investigated. Figure 3 shows the growth curves of the NHEKs cultured in the indicated medium. Until day 20, the cells grown in the media containing 0.001% and 0.003% MP proliferated at the same rate as that of the control. Thereafter, the proliferating ability of the MP-treated cells decreased when compared with that of the control, and their population doubled fewer times than the control population.

Apoptosis of NHEKs

The effect of low-concentration MP exposure on apoptosis of NHEKs was investigated. Apoptotic and necrotic cells were detected by staining with annexin V and propidium iodide (PI), respectively. The positive cells were

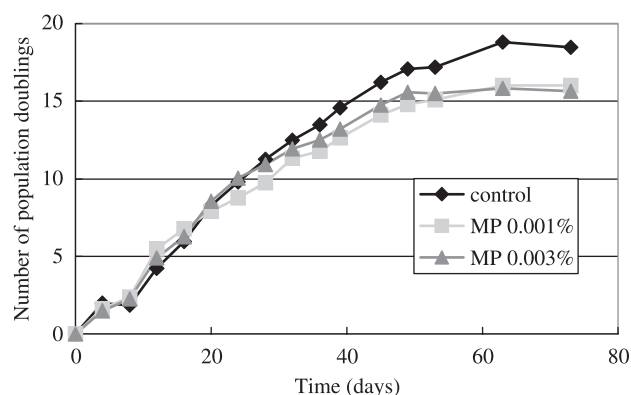


Figure 3. Effect of long-term exposure to MP on NHEK growth. Representative growth curves of ketatinocytes under the indicated culture conditions. Each value represents the average number of population doublings ($n = 2$)

counted. Annexin V binds phosphatidylserine (PS) with high affinity. PS translocation from the inner to the external leaflet of the plasma membrane is considered as one of the earliest events in apoptosis. Until 16 days after treatment, apoptotic and necrotic cells were not detected. However, the MP treatment resulted in an increase in both annexin V-positive and PI-positive cells at 32 days (Fig. 4).

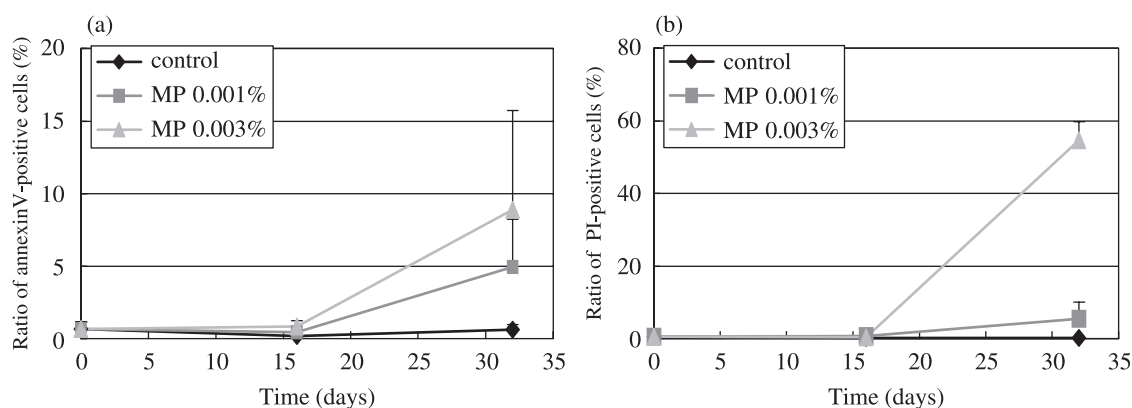


Figure 4. Ratio of apoptosis and necrosis induced by MP in NHEKs. Each value (mean $\pm$ SD) represents the ratio of (a) apoptotic cells and (b) necrotic cells

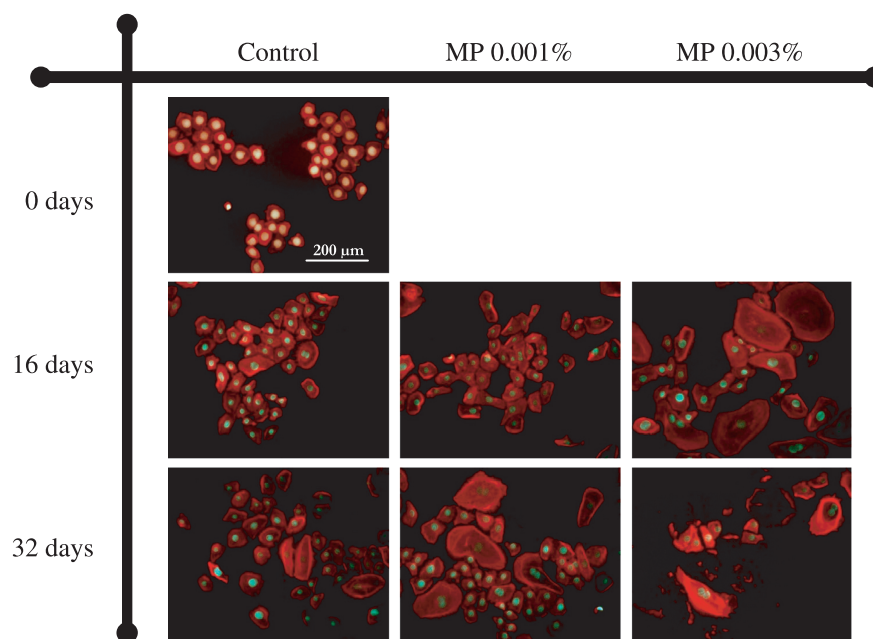


Figure 5. Morphological changes induced by MP in NHEKs. F-actin was labeled by Alexa Fluor® 568-conjugated phalloidin staining (red fluorescence). The nuclei were labeled by DAPI staining (blue fluorescence). Original magnification, 400 $\times$.

Morphological Change in NHEKs

Figure 5 shows the morphological change in NHEKs based on the culture period and MP treatment. The cells were enlarged and flattened. These characteristics of cellular aging were accelerated by the addition of MP.

Effects on the Expressions of mRNA and Protein

To evaluate the effects of MP on NHEKs in greater detail, the mRNA levels of several types of genes were estimated. The mRNA expression levels of hyaluronan synthases (HAS) 1 and 2 in NHEKs decreased after MP application. HAS3 gene expression was not influenced by MP application (Fig. 6).

These cells were then analysed by Western blotting. Type IV collagen expression was decreased by MP application. In contrast, MP application increased HSP27 and involucrin expressions (Fig. 7).

Effects of MP on the Differentiation of Epidermis

To examine the effect of proliferation and differentiation of the epidermis of the skin equivalent, the equivalents were cultured in a medium with or without 0.003% MP. The morphology of skin equivalent is shown in Fig. 8a.

H&E staining demonstrated that several flattened layers formed the granular layer, which contained keratohyalin

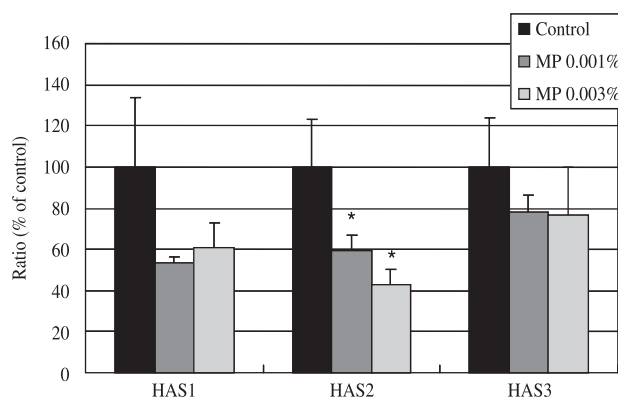


Figure 6. Hyaluronan synthase mRNA expression in MP-treated NHEKs. Each value (mean $\pm$ SD) represents the ratio of HAS1, HAS2 and HAS3 mRNA expressions to control. Asterisks indicate significant differences (* $P < 0.05$) from control

granules beneath the SC in both equivalents. However, fewer cells contained the keratohyalin granules in the equivalent cultured with MP, and there were some nucleated cells in the SC of the equivalent cultured with MP. No obvious differences in SC thickness were observed between control and the equivalent cultured in the medium containing 0.003% MP. For the quantitative comparison of the skin equivalents cultured with and without MP, the selected protein marker HSP27 was determined by Western blotting. HSP27 expression was increased in the skin equivalent cultured with MP (Fig. 8b).

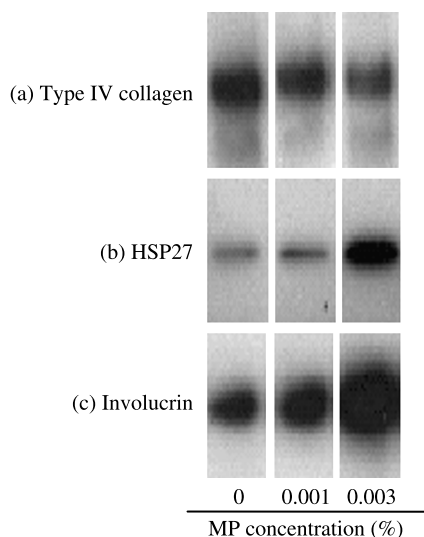


Figure 7. The expressions of type IV collagen, HSP27 and involucrin in MP-treated NHEKs. Proteins from control and MP-treated NHEKs were subjected to Western blotting

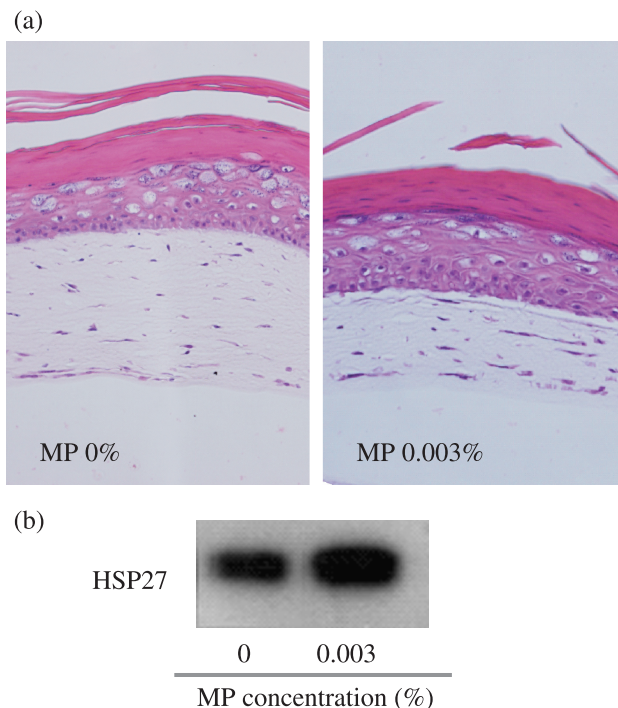


Figure 8. Effects of MP on skin equivalent. (a) Histology of skin equivalent: skin equivalents were cultured with the medium containing or not containing MP and subsequently subjected to H&E staining. Original magnification, 200 $\times$. (b) Western blot analysis of HSP27 in the skin equivalent: skin equivalents were cultured with the medium containing or not containing MP and were analysed by Western blotting for HSP27.

Discussion

The results of our experiments suggest that MP is not hydrolysed completely and retained in the skin after daily topical application of MP containing formulations. These are important data for discussing the effects of MP on skin.

There were some reports on the percutaneous absorption of parabens after a single application of various formulations on human skin *in vitro* (Dal Pozzo and Pastori, 1996; Cross and Roberts, 2000). Using human skin, Dal Pozzo and Pastori (1996) studied the percutaneous absorption of a series of six parabens, and noted their subcutaneous penetration was increased with increasing lipophilicity. Cross and Roberts (2000) also determined the effect of occlusion on the epidermal penetration of parabens. However, these experiments were conducted using high concentrations of parabens in the diffusion cells, and it is difficult to interpret these data in terms of the actual exposure to formulations. The absorption and accumulation of parabens in the skin has not been well investigated in the case of constant dermatological exposure, for example, use of cosmetic formulations.

This study evaluated the influence of daily exposure to MP by applying MP containing formulations to human skin. The MP concentration in the SC was measured after daily treatment with MP containing formulations. The MP concentrations increased in accordance with the duration of formulation use. This result indicates that MP applied to the skin is not completely metabolized, and a part of it remains and accumulates in the SC of human skin during continuing use of dermatological formulations. In fact, 12 h after a single application, MP concentrations were about 10 pmol cm⁻² (approximately 0.028% of the application quantity of MP). Subjects used formulations twice a day, so they used them every 12 h. This means that subjects re-applied the formulations before the MP in the SC had been absorbed and metabolized completely. If this procedure continued, MP would accumulate in the SC. By contrast, after the subjects stopped using the formulations, the MP concentrations decreased to the baseline level immediately. This may indicate that the rate of MP hydrolysis was slower than the rate of MP application, which was performed twice a day. Lobemeier *et al.* (1996) reported that in humans, the specific activity of carboxylesterases capable of hydrolysing parabens was lower in the carboxylesterase from the whole skin extract than in those from the subcutaneous fatty tissue. Our results suggest that continuous application results in MP remaining in the skin when MP hydrolysis occurs at a slow rate on the surface of the skin.

As mentioned above, repeated application increased the MP concentrations in the SC. In considering the daily and common use of cosmetic formulations, two groups were designed. Subjects of one group applied only lotion, while the subjects of the other group used both lotion and

emulsion. Both groups noted the same tendency. However, the MP concentration in the SC of those subjects who used both lotion and emulsion was higher than in the SC of the subjects who used only lotion. This suggests that MP accumulation in the SC increases with sustained use of MP containing formulations, such as by their continuous topical application, until MP is saturated in the skin. However, we were unable to determine the time required for the saturation of MP in this experiment; further investigations will be required to clarify the saturation point of MP due to continuous topical application.

The concentrations of MP in the SC, epidermis and dermis were investigated by employing the epidermal diffusion system using the Yucatan micropig skin samples *in vitro*. These skin samples are often used for skin penetration studies because they are similar to human skin with regard to permeability (Fujii *et al.*, 1997; Lavker *et al.*, 1991; Rohatagi *et al.*, 1997). The MP concentration in the epidermis peaked to $19.3 \mu\text{g g}^{-1}$ tissue, that is 0.002% of tissue weight in percent figures, at 1 h and slightly decreased to $15.0 \mu\text{g g}^{-1}$ tissue at 2 h. Cross and Roberts (2000) showed that a similar MP concentration in the human epidermis remained at 10 h after a single exposure.

On the other hand, the epidermal concentration of PHBA, a paraben metabolite, increased sequentially. Although the difference in MP hydrolysis between human skin and the Yucatan micropig skin could not be clarified, our result suggests that the MP concentration in the SC kept increasing by repeated application. The MP concentration can reach approximately 0.002% of tissue weight temporarily after every application of MP containing formulations. This result was used to investigate the effects of MP on NHEKs.

Based on the above data, the effects of long-term MP exposure on NHEKs was next investigated *in vitro*. Accordingly, 0.001%–0.003% MP concentrations were used. Additionally, acute cytotoxicity potential was measured by the MTT assay method (24 h exposure). The EC_{50} value was 0.04% (w/v) (data not shown). Considering this result, the MP concentrations (0.001%–0.003%) that were used to evaluate the effects of MP on NHEKs were low and cannot induce acute cytotoxicity.

At the beginning, the effect of MP on NHEK growth curves was examined. The cells grown in the medium containing MP had lower proliferative potential than the control cells. We tested whether growth in such medium was associated with a high rate of apoptosis. Until 16 days, no difference was observed between the growth of the cells grown in the medium containing MP and that of the control cells, and apoptotic or necrotic cells were hardly detected. At 32 days, the growth of the cells grown in the medium containing MP was lower than that of the control cells, and both apoptotic and necrotic cells in the medium containing MP increased in a dose-dependent manner. Thus, the rate of apoptosis might have

a relationship with the poor growth of the MP-treated cells. Additionally, the cells grown in the medium containing MP became enlarged and flattened.

There are many reports regarding the relationship of the senescence or cellular changes and repeated subculture. Cells that undergo repeated subculture have often been used as a cellular senescence model (Youn *et al.*, 2004; Bernard *et al.*, 2004). Normal cells have a limited proliferative lifespan after which they arrest and lose the ability to divide. This phenomenon is termed replicative senescence and is thought to contribute to tissue aging (Gandarillas, 2000). Early-passage newborn human keratinocytes maintained in a serum-free system were found to proliferate more rapidly than early-passage adult keratinocytes maintained under identical conditions (Gilchrest, 1983). Human senescent cells display the characteristic enlarged and flattened morphology (Angello *et al.*, 1989; Norsgaard *et al.*, 1996). In this study, MP delayed the growth of NHEKs. In addition, it accelerated the morphological changes of NHEKs, which occurred with repeated subculture and increased the apoptotic cells. This suggests that MP acts as an effector of the senescent phenotype of NHEKs.

Since these results suggest the possibility that MP accelerated the senescence of NHEKs, the effect of MP was evaluated on HAS and type IV collagen. Hyaluronan, synthesized by HAS enzymes, is a large glycosaminoglycan found in the extracellular space of most animal tissues and is essential for cell proliferation and migration. Previous studies have noted that hyaluronan declines with aging (Fleischmajer *et al.*, 1972; Longas *et al.*, 1987), which accounts for the deterioration of the aging skin. Type IV collagen, which is a basement membrane component, also declines with aging (Vazquez *et al.*, 1996). MP treatment reduced the expressions of the HAS1 and HAS2 mRNAs as well as type IV collagen. These results may suggest that MP is related to the aging of NHEKs. However, it is necessary to evaluate a senescence marker, for example, telomere length and β -galactosidase activity.

Additionally, it was revealed that MP increased involucrin and HSP27 expression. Involucrin is a differentiation marker of NHEKs. Involucrin expression initiates in the early spinous zone and is maintained in the granular layer, and it is cross-linked early in cornified envelope formation (Robinson *et al.*, 1996). HSP27, the 27 kDa heat shock protein, belongs to a group of small heat shock proteins. It plays a protective role during stress and has a variety of functions as a molecular chaperone (Jakob *et al.*, 1993). HSP27 expression has been correlated with and can be regarded as a marker of NHEK differentiation (Kindas-Mugge and Trautinger, 1994). Hyaluronan is also involved in the proliferation and differentiation of epidermis (Pasonen-Seppanen *et al.*, 2003). Our results suggest that MP also affects the differentiation of NHEKs.

Furthermore, in order to evaluate the effect of MP on skin conformation, the skin equivalents were cultured with or without MP for 1 week from day 7 until day 14. The cellular viability of the skin equivalents was determined by the MTT assay. No difference in cell viability was observed between the skin equivalent cultured with and that cultured without MP (data not shown). However, in the skin equivalent cultured with MP, some histological differences were observed and upregulation of the selected protein marker HSP27, which is involved in the regulation of cell growth and differentiation, was confirmed by Western blotting analysis. These data indicate that low MP concentration might affect the keratinocyte differentiation process, generating an abnormal disorganized epidermis.

In summary, in order to clarify the effects of MP on human skin, particularly on the SC and epidermis, the MP concentration in the SC was measured during long-term application of MP containing formulations. One month of twice-daily applications significantly increased the MP concentration in the SC. This indicates that MP is not metabolized completely and persists slightly in the SC, and its concentration increasing with prolonged application of MP containing formulations. Therefore the effects of MP on keratinocytes were evaluated. MP accelerated some changes in keratinocytes that occur with repeated subcultures. MP decreased the expressions of HAS1 and HAS2 mRNAs as well as type IV collagen but increased the expressions of HSP27 and involucrin. Furthermore, MP affected the development of the epidermis in the skin equivalent. These results suggest that MP accumulated in the skin through the daily use of MP containing formulation may be related to the differentiation and aging of skin keratinocyte. Further investigations, particularly *in vivo* tests, are required for a more precise understanding.

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