Negative Electric Potential Induces Alteration of Ion Gradient and Lamellar Body Secretion in the Epidermis, and Accelerates Skin Barrier Recovery After Barrier Disruption

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Previous reports suggested that ion gradients of ions such as calcium and magnesium in the epidermis play a crucial part in skin barrier homeostasis. We hypothesized that external electric potential affects the ionic gradient and skin barrier homeostasis. We demonstrated here that application of a negative electric potential (0.50 V) on hairless mice skin accelerated the barrier recovery approximately 60.7% of the original level within 1 h compared with control (37.5%) after barrier disruption by acetone treatment. Even after the application of a negative potential, the skin showed accelerated repair for 6 h. On the contrary, the skin that was applied a positive potential for 1 h showed a significant delay in barrier recovery (25.3%) than the control. Ultrastructural studies by electron microscopy suggested that the extent of lamellar body exocytosis into the stratum corneum/stratum granulosum interface increased under a negative potential. Magnesium and calcium ion concentrations in the upper epidermis were relatively higher in the negative portion than in the portion where the positive potential was applied. Topical application of these ions on mice skin also accelerated the barrier recovery. These results suggest that the external electric potential affects the ionic gradients in the epidermis and also influences the skin barrier homeostasis. ***Key words: stratum corneum/keratinocyte/magnesium/calcium. J Invest Dermatol 118:65–72, 2002***

One of the most important roles of the skin is to generate a water-impermeable barrier against excess transcutaneous water loss. In common dermatoses, such as atopic dermatitis (Werner and Lindberg, 1985) and psoriasis (Tagami and Yoshikuni, 1985), a decline in barrier function often parallels increased severity of clinical symptomatology. This barrier function resides in the uppermost thin layer called the stratum corneum (SC). The SC is composed of two components, i.e., protein-rich nonviable cells and intercellular lipid domains (Elias and Menon, 1991). When the SC barrier function is damaged by tape stripping or treatment with an organic solvent or detergent, a series of homeostatic processes in barrier function is immediately accelerated, and the barrier recovers to its original level (Elias and Menon, 1991). This process includes lipid synthesis, lipid processing, and the acceleration of exocytosis of lipid-containing lamellar bodies in the upper epidermis. Previous reports suggested that localization of ions such as calcium (Lee *et al*, 1992; Mauro *et al*, 1998) and magnesium (Denda *et al*, 1999, 2000) play a crucial part in barrier homeostasis. Topical application of calcium chloride solution delayed barrier repair after barrier disruption (Lee *et al*, 1992). On the other hand, the application of some magnesium salts or mixture of calcium and magnesium salts accelerated barrier recovery 3 h after barrier damage (Denda *et al*, 1999). In normal skin, both calcium and magnesium were localized in the outermost epidermis [(stratum granulosum (SG)], and within 30 min after barrier disruption, the gradation of calcium (Mauro *et al*, 1998; Denda *et al*, 2000) and magnesium disappeared (Denda *et al*, 2000). Normal skin surface has a negative potential against the inside and the potential decreases immediately after barrier disruption (Edelberg, 1977). Barker *et al* (1982) suggested that the potential was induced by epidermal living layers. Alteration of ion localization changes the electric potential (Mitchell, 1966). From these studies, we hypothesized that there is a correlation between skin surface electric potential and ion localization in the epidermis. We also expected that an external electric potential affects the ion gradation in the epidermis and as a result, skin barrier function can be regulated by the potential. In this study we applied electric potential and demonstrated their effect on epidermal barrier repair after acetone treatment and also performed ultrastructural studies on skin barrier homeostasis under positive and negative electric potentials. The findings suggest a new method of improving cutaneous barrier function within a short period of time by external electric potential without topical application of any bioactive chemical materials.

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Abbreviations: SC, stratum corneum; SG, stratum granulosum; TEWL, transepidermal water loss.

MATERIALS AND METHODS

Animals Hairless mice, 7–10 wk old (HR-1, Hoshino, Yashio-Saitama, Japan) were used. Before the experiments, animals were caged separately for at least 4 d. These cages were maintained in a room kept at a temperature of 22–25°C and a relative humidity of 40–70%. All experiments were approved by the Animal Research Committee of the

Shiseido Research Center in accordance with the National Research Council Guide (1996).

Application of electric potential Immediately after barrier disruption by acetone treatment both sides of the flank skin of mice under anesthesia, a pair of 2 × 2cm pure gold plates electrode was attached. A constant DC voltage (0.50 V) was loaded by a function generator (Type 19, Wavelike, Ft Worth, TX) for 1 h. The side of the flank on which an negative electrode was attached, was defined as a negative potential loading area and the other side of the flank in which a positive electrode was attached, was defined as the positive potential loading area. An area that was treated with acetone and was not attached to the gold plate was defined as the control without potential loading. During the application of potential, the electric current was monitored with a digital multimeter (System Digital Multimeter, type 200, Keithley, Cleveland, OH).

Barrier function and recovery kinetics Skin barrier function was evaluated by measuring transepidermal water loss (TEWL), using an electrolytic water analyzer (Meeco, Warrington, PA), as described previously (Denda *et al*, 1999). For barrier recovery experiments, both sides of the flank skin were treated with repeated application of acetone soaked cotton balls until the TEWL reached 7–10 mg per cm^2 per h, as described previously. In this study, we did not use tape stripping for barrier disruption because it heterogeneously damages the barrier and consequently, the electric current might also become heterogeneous. TEWL was then measured over the same sites at 1, 3, and 6 h after barrier disruption. The barrier recovery results are expressed as percent of recovery, because of variations from day to day in the extent of barrier disruption. In each animal, the percentage of recovery was calculated by the following formula: (TEWL immediately after barrier disruption − TEWL at indicated time point)/(TEWL immediately after barrier disruption − baseline TEWL) × 100%.

Light and electron microscopy For the series of histology studies, we used animals different from those used in the barrier kinetics experiment. Immediately after the end of the potential application, mice were killed and skin samples were isolated. Skin samples for fluorescence microscopy were embedded in OCT compound, sectioned and 4 mM Nile Red (100 μg per ml, 75% glycerol) was applied. Sections were examined with a microscope (Vanox AHBT3, Olympus) equipped for epifluorescence at an excitation of 470–490 nm and emission of 520 nm (Denda *et al*, 1998a). For the observation, at least five sections were observed to find common features.

The full thickness of skin samples for electron microscopy was minced into pieces (< 0.5 mm^3) and fixed in modified Karnovsky's fixative overnight. They were then postfixed in 2% aqueous osmium tetroxide or 0.2% ruthenium tetroxide as described previously (Ghadially *et al*, 1995). After fixation, all samples were dehydrated in graded ethanol solutions, and embedded in an Epon-epoxy mixture. Thin sections were stained with lead citrate and uranyl acetate and viewed by electron microscopy (Ghadially *et al*, 1995). The area of the SC/SG lipid domain and number of free lamellar bodies were quantified from osmium postfixed material. Measurements were made without knowledge of the prior experimental treatment. These parameters were evaluated from photographs of randomly selected sections at a constant magnification, using computer software (NIH image). The free lamellar bodies were defined as the granules that were localized within 2 μm from the SC/SG interface and not attached to the SC/SG interface.

Visualization of ion localization Previously, we demonstrated that both calcium and magnesium played an important part in skin barrier recovery (Denda *et al*, 1999). Thus, we hypothesized that the application of electric potential affects the gradation of these ions. We observed the localization of calcium and magnesium using a method reported previously (Denda *et al*, 2000). For each observation, at least five sections were observed to find common features. Calcium Green 1 and Magnesium Green were purchased from Molecular Probes (Eugene, OR). Agarose gel (final 2%) containing each indicator was spread on the slide glass until 50 μm thick. To observe calcium, Calcium Green 1 at a final concentration of 10 μg per ml was mixed before the formation of the membrane. In order to observe magnesium, Magnesium Green at a final concentration of 10 μg per ml and ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid at a final concentration of 0.2 mM were mixed together. The frozen section (5 μm in thickness) was put on the gel membrane and within a couple of hours, the whole picture was taken.

Topical application of salt mixture Previously (Denda *et al*, 1999), we demonstrated that topical application of the calcium and magnesium chloride mixture accelerated skin barrier repair 3 h after barrier disruption. To confirm the effect of the salt mixture within a shorter period of time, i.e., 1 h after barrier disruption, we carried out the following experiment. Magnesium chloride and calcium chloride were purchased from Wako (Osaka, Japan). Immediately after barrier disruption with acetone treatment, 50 μl of aqueous solution was applied and the area was occluded for 10 min with a plastic membrane (Saran; Dow Chemical, IN) 2 × 3 cm^2, which was then removed. In a previous study (Denda *et al*, 1999), we applied water on the control side; however, in this study, the control area was treated with acetone at the same level and did not apply any treatment to avoid the effect of water or occlusion.

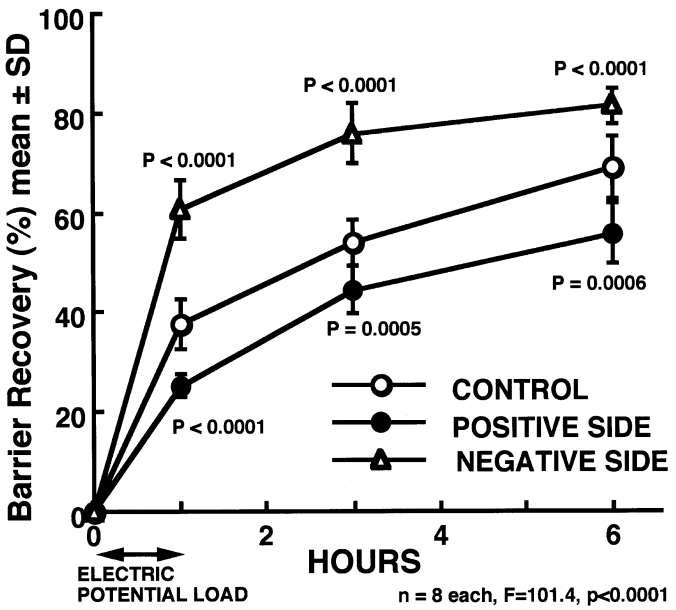

Figure 1. Electric potential affects the skin barrier recovery rate. Immediately after the end of the electric potential loading, the negative side showed approximately 60.7% of recovery to the original level, which was significantly higher than that on the control side without the potential load (37.5%). At 3 and 6 h after barrier disruption, the recovery rates were significantly higher on the negative side than on the control side even without the electric load (75.8 and 81.5%, respectively, at 3 and 6 h on the negative side and 53.9 and 69.0%, respectively, on the control side). On the other hand, the positive side showed slower barrier repair at each time point than the control (25.3, 44.3, and 55.8% at 1, 3, and 6 h, respectively). Numbers of subjects and the results of statistical analysis are shown in the figure.

Statistics The results are expressed as the mean ± SD. Statistical differences between two groups were determined by a two-tailed Student's t test. In the case of more than two groups, differences were determined by ANOVA test (Fisher's protected least significant difference).

RESULTS

The recovery time course of the barrier function after acetone treatment under a positive and negative potential is shown in **Fig 1**. When we applied 0.10 V, the electric current was too small to detect, and application of more than 1.0 V might cause electrolysis; therefore, in this study we applied 0.50 V.

Immediately after the end of the electric potential application, the negative side showed approximately 60.7% recovery of the original level, which was significantly greater than that on the control side without potential loading (37.5%). At 3 and 6 h after barrier disruption, the recovery rates were significantly higher on the negative side than on the control side even without electric loading. On the other hand, the positive side showed slower barrier repair at each time point than the control. Within 1 h after barrier disruption, the barrier repair rate significantly altered under different electric potential. In this study, the control side was not occluded by the gold plate. Previously, occlusion with water

impermeable material was found to block barrier repair after its damage (Grubauer *et al*, 1989). Thus, the delay of barrier repair in the positive potential area might be due to the occlusion. If the gold plate had an occluding effect, the barrier recovery effect of the negative potential in our study, overwhelmed the occlusive effect.

As shown in **Table I**, the electric current significantly decreased during the potential application. This means an increase of electric resistance during the potential loading as shown in **Table I**. The high resistance suggested that the small electric current fluxed via skin. The Nile Red staining immediately after the end of potential application showed a recovery of lipids in the upper epidermis on the negative side (**Fig 2*A***, red fluorescence, *arrows*), although lipid recovery was not so obvious on either the positive or control side at the same time (**Figs 2*B***, ***C***). Hematoxylin and eosin stained sections of hairless mouse skin that were treated by acetone the same as the other sections are shown in **Fig 2*D***. As shown in **Fig 3**, an ultrastructural study by osmium-fixed electron microscopy revealed a thick secreted lipid domain between the SC and SG on the negative side (**Fig 3*A*** *arrows*) and very few lamellar bodies were observed in the SG. These changes were not observed on either the positive or control side (**Figs 3*B***, ***C***). **Figure 4** shows a ruthenium-fixed sample from the negative side skin immediately after the end of potential application. An abnormally large number of secreted lamellar bodies was observed between the SC and the SG (*arrows*). The results of quantification of the ultrastructural studies are shown in **Fig 5**. The area of secreted lipid domain between the SC and the SG per 4 μm was significantly higher on the negative side than on any other part or the untreated control (**Fig 5*A***). On the other hand, the number of lamellar bodies per 8 μm (2 μm from SC/SG × 4 μm wide) of upper epidermis was significantly lower on the negative side than in any other sample (**Fig 5*B***). Localization of calcium and magnesium immediately after a 1 h potential application is shown in **Fig 6**. These are the representatives of each observation. On the negative side, high concentrations of both calcium (**Fig 6*A***) and magnesium ions (**Fig 6*C***) were observed in the uppermost epidermis. Localization of the ions in the uppermost epidermis was not so obvious on the positive side (**Figs 6*B***, ***D***). Hematoxylin and eosin stained sections of hairless mouse that were treated by acetone in the same way as the other sections are shown in **Fig 6**(***E***). In a previous study of human skin, the highest concentration of calcium and magnesium was observed in the SG (Denda *et al*, 2000). In the frozen section of hairless mouse skin, it was difficult to distinguish the SG from the SC because of the thinner SC of the hairless mouse than that of human skin. The high concentrations of calcium and magnesium in the uppermost epidermis were commonly observed in five different sections. These results suggest that an external electric potential affected the localization of ions in the epidermis. We previously demonstrated that a topical application of 10 mM calcium chloride and magnesium chloride mixture accelerated barrier repair 3 h after barrier disruption (Denda *et al*, 1999). In the present study, we evaluated the effect of application of the mixture 1 h after barrier disruption. As shown in **Fig 7**, the mixture accelerated barrier recovery within 1 h in a concentration-dependent manner.

Table I. Electric current before and after application of a DC potential (0.5 V) load

	Before the load	After the load
Electric current	0.68 μA ± 0.23	0.21 μA ± 0.03
	(p = 0.0017)	
Calculated resistance	735 kΩ	2381 kΩ

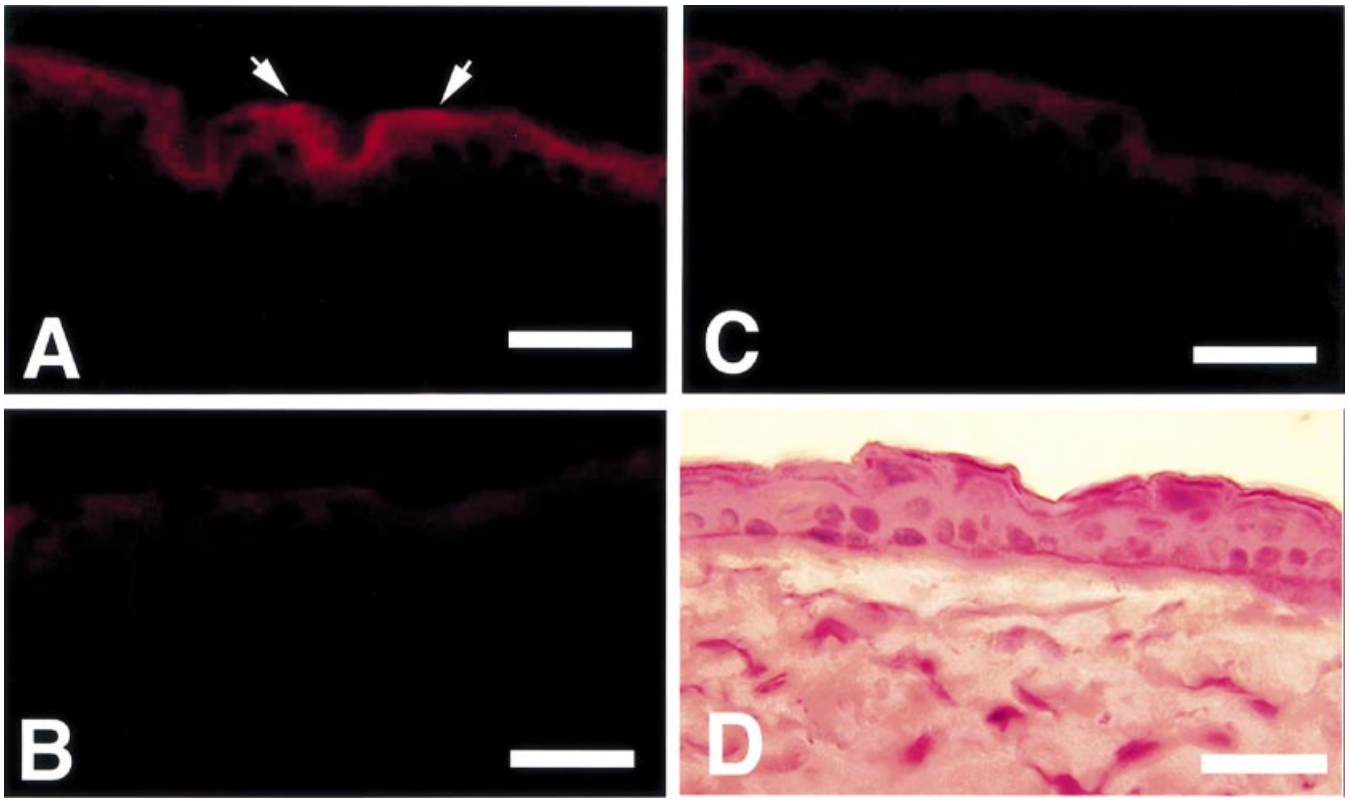

Figure 2. A negative potential induces lipid deposition. Intense red (lipid: *arrow*) membrane staining is seen in the outermost epidermis on the negative side (*A*), although lipid recovery was not so obvious on either the positive or control side (*B,C*). Each photograph is representative of n = 5. Hematoxylin and eosin stained section of the hairless mouse skin that was treated with acetone the same as other sections is shown in (*D*). *Scale bars*: 10 μm.

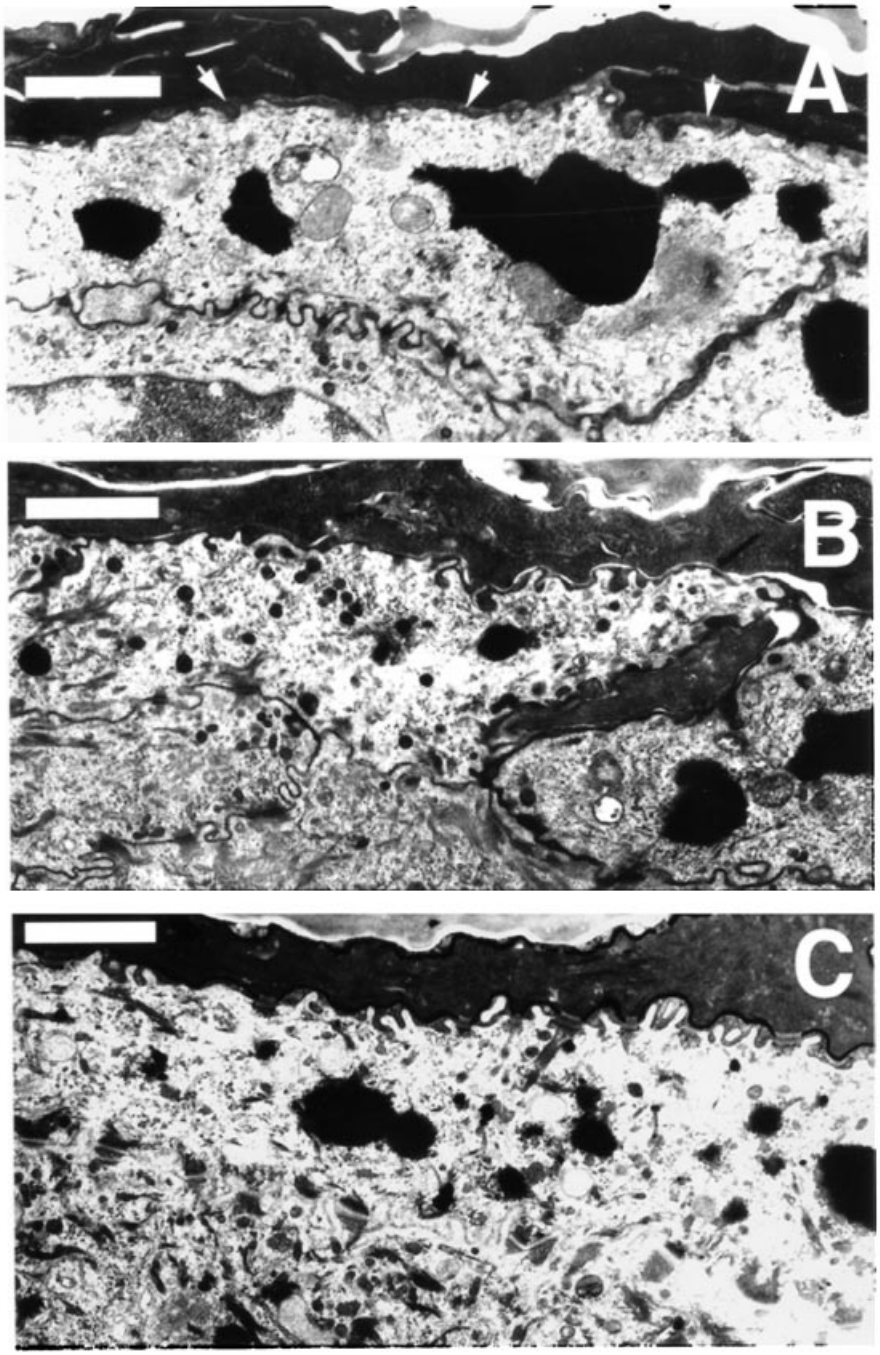

Figure 3. A negative potential induces lamellar body secretion. There was a thick secreted lipid domain between the SC and SG on the negative side (*A*, *arrows*) and very few lamellar bodies were observed in the SG. Such changes were not observed on either the positive or control side (*B*, *C*). The samples were postfixed by osmium tetroxide. *Scale bars*: 1 μm.

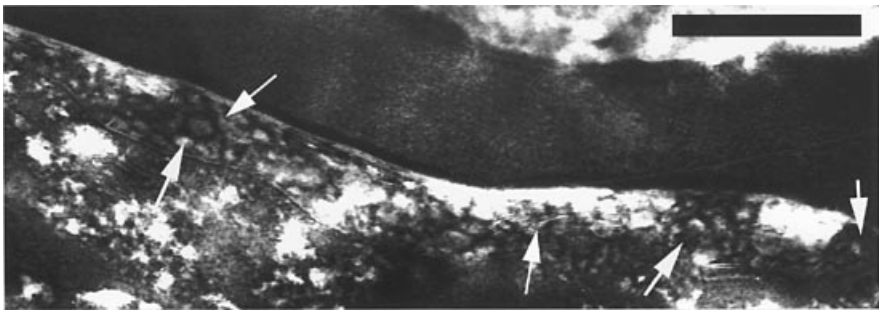

Figure 4. An increase of lamellar body secretion was observed between the SC and SG (*arrows*) on the negative side. Sample was postfixed by ruthenium tetroxide. *Scale bar*: 0.5 μm.

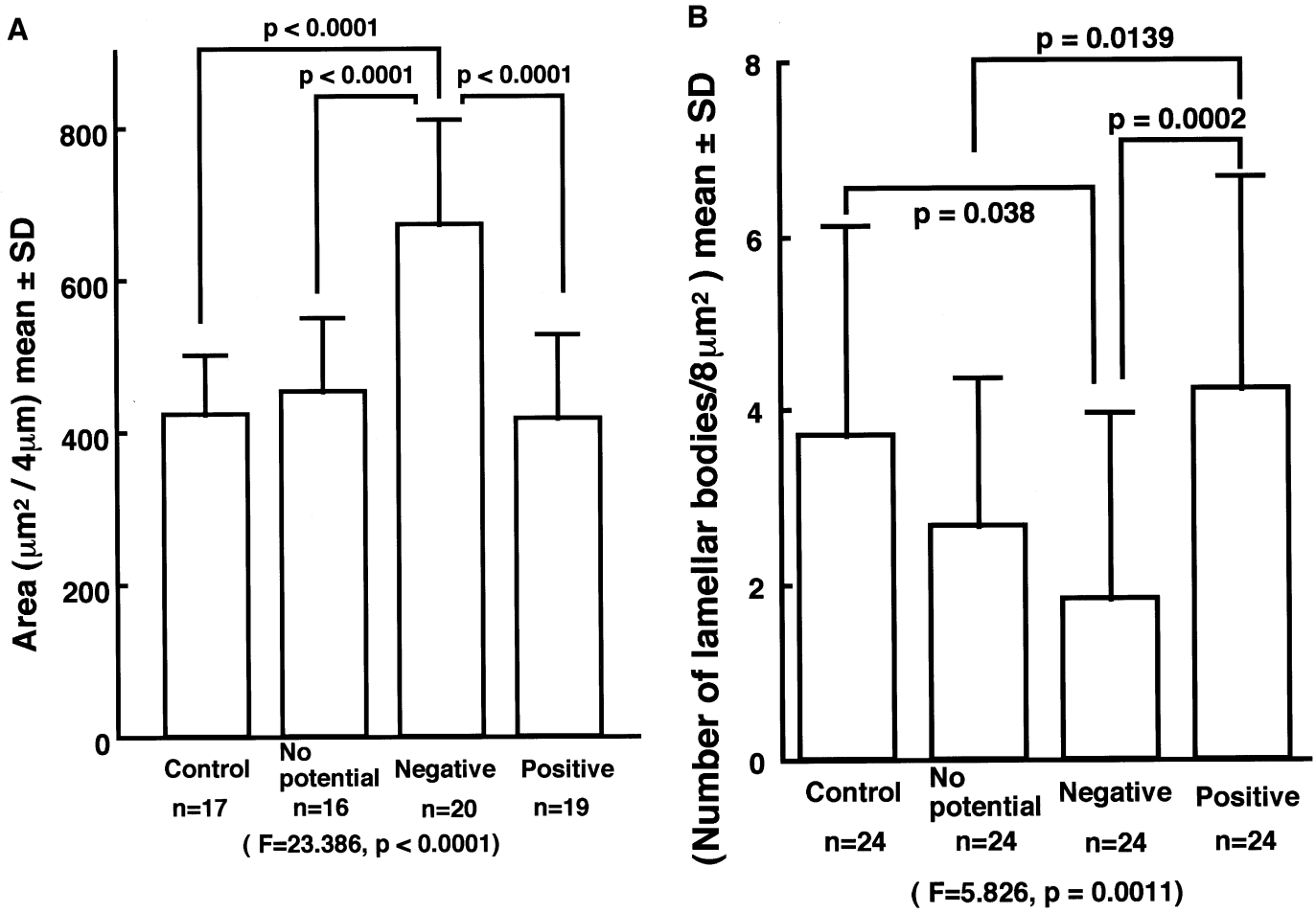

Figure 5. The area of secreted lipid domain between the SC and SG per 4 μm was significantly higher on the negative side than the other parts and untreated control (*A*). On the other hand, the number of lamellar bodies per 8 μm of the uppermost epidermis (2 μm depth from SC/SG interface × 4 μm wide) was significantly lower on the negative side (*B*) than in any other sample. 'n' is the number of sections for the quantification of the data.

DISCUSSION

Previously, Lee *et al* (1998) demonstrated that iontophoresis on hairless mouse skin induced the loss of epidermal calcium gradient; however, they did not observe any alteration in the basal TEWL of the skin. In our study, we applied potential immediately after barrier disruption and evaluated the effect of potential on the barrier repair process. The external potential might only affect the barrier homeostatic process in the epidermis. Lee *et al* (1998) also applied three drops of water on to the skin where they attached the electrode (the applied voltage was 6 V). We did not apply any water between the skin and electrode in this study and a lower voltage (0.50 V) was applied to avoid electrolysis of water. These differences might also affect epidermal barrier function differently.

In this present study, we loaded an electric potential externally on the skin; however, the mammalian skin surface usually has a negative electric potential. Barker *et al* (1982) demonstrated the existence of a high electric potential on the epidermis in humans and guinea pigs. They reported that amiloride decreased the potential and suggested that the potential was in the living, not dead, cell layers of the epidermis; however, the mechanism of the endogenous voltage in the epidermis has not been clarified. The skin surface potential is reduced by disruption of the SC with tape stripping (Edelberg, 1977). Recently, fast movement of ions such as calcium, potassium, and magnesium in human epidermis (Denda *et al*, 2000) and hairless mice epidermis (Mauro *et al*, 1998) immediately after disruption of the SC has been reported. In normal human skin, both calcium and magnesium are localized in the granular layer, i.e., the outermost layer of the epidermis (Denda *et al*, 2000). The gradation of ions might induce the skin surface potential, but the role of the skin surface potential has not been clarified yet.

Previous reports suggested the efficacy of an external electric current on skin wound healing. Alvarez *et al* (1983) demonstrated that a 50–300 μA, DC below 0.9 V accelerated the epithelialization of a 0.3 mm deep wound on pig skin. Previous studies have demonstrated the effect of a DC electric field on keratinocyte migration (Nishimura *et al*, 1996; Sheridan *et al*, 1996). They reported that under an electric field of approximately 100 mV per mm, cultured human keratinocytes migrated toward the negative pole. These results suggest that the keratinocyte is influenced by the environmental electric field on its physiologic responses.

Alteration of ionic gradation by external potential might be due to an electrostatic interaction between negative electrodes and cations in the epidermis. Ion channels are also influenced by the external electric potential (Chernyak, 1995). Therefore, electrophysiologic features of keratinocytes should be investigated further.

Acute disruption of the skin barrier by tape stripping or treatment with an organic solvent or detergent, elicits a homeostatic response in the epidermis that rapidly results in the restoration of barrier function. In addition, acute disruption of the barrier results in an increase in epidermal DNA synthesis (Proksch *et al*, 1991) and cytokine production (Wood *et al*, 1992). Even the damage of the barrier is relatively small, when it is repeated (Denda

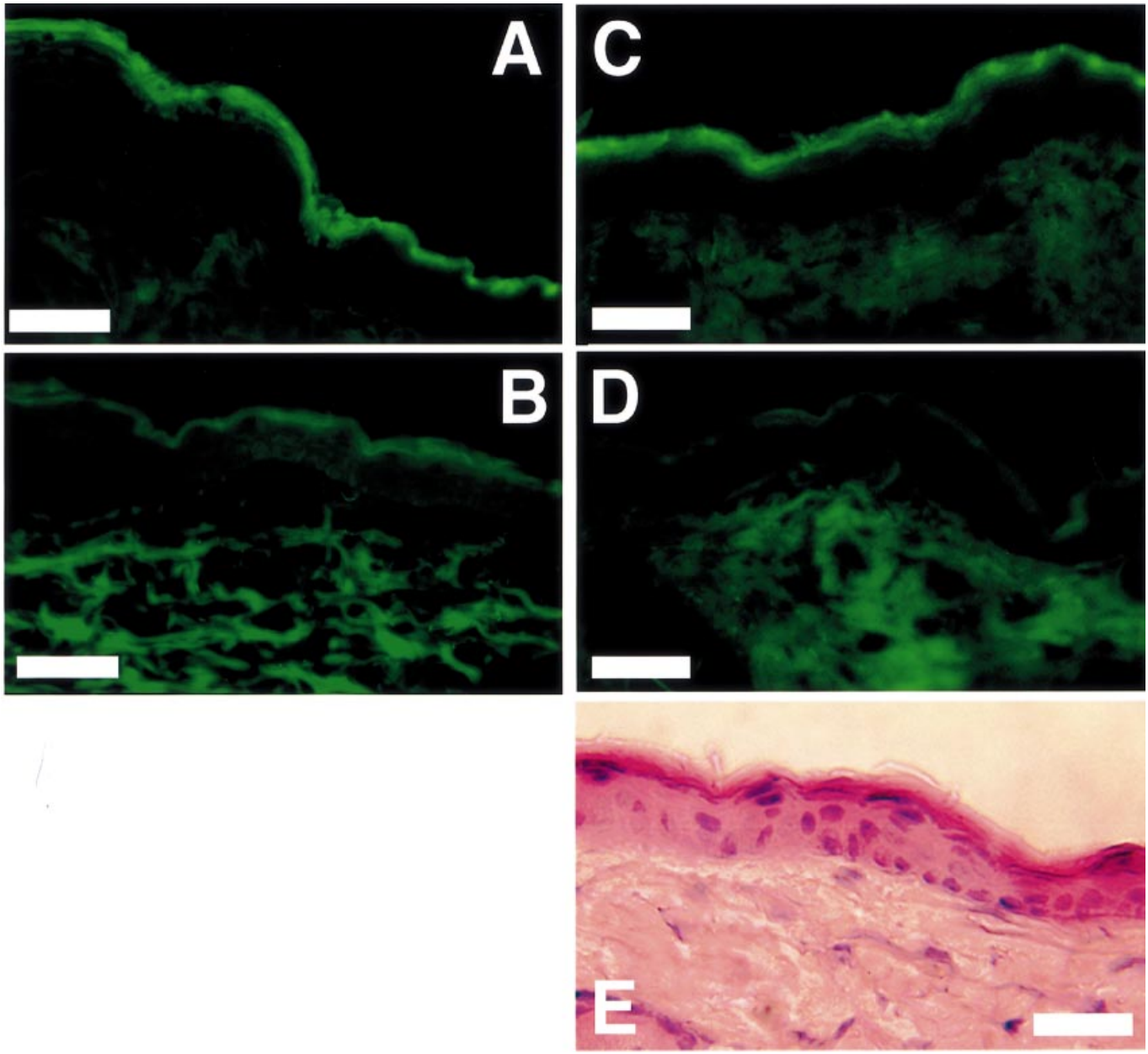

Figure 6. On the negative side, high concentrations of both calcium (*A*) and magnesium ions (*C*) were observed in the SC and SG. The localization of the ions was not so obvious on the positive side (*B*, *D*). Each photograph is representative of n = 5. Hematoxylin and eosin stained section of the hairless mouse skin that was treated with acetone that was the same as other sections was shown in (*E*). *Scale bars*: 10 μm.

et al, 1996), or under low environmental humidity (Denda *et al*, 1998b) the damage induces an obvious epidermal hyperplasia and inflammation. The mechanism of the signal transfer after barrier disruption remains unclear, but ions such as calcium and potassium have been suggested to have a significant role in keratinocyte differentiation or barrier homeostasis (Lee *et al*, 1992; Clapham, 1995). The drastic movement of ions or the alteration of the electric potential after the barrier insult might be an important signal for the homeostatic process.

Calcium plays various parts in SC barrier formation (Menon *et al*, 1992). For example, it induces terminal differentiation (Watt, 1989), formation of the cornified envelope, and also epidermal lipid synthesis (Watanabe *et al*, 1998). Menon *et al* (1994) demonstrated that alteration of the calcium gradient affects the exocytosis of the lamellar body to the interface between the SC and the epidermal granular layer. Vicanova *et al* (1998) demonstrated the improvement of barrier function of the reconstructed human epidermis by the normalization of epidermal calcium distribution. Magnesium is required for the activity of Rab-geranylgeranyl transferase, which modifies Rab (Seabra *et al*, 1992). After modification, Rab plays an important part in exocytosis and endocytosis (Novic and Brennward, 1993). For barrier formation, exocytosis of the lamellar body is an important process. Previous studies have indicated that Rab is modified by Rab-geranylgeranyl transferase during the terminal differentiation of the epidermis (Song *et al*, 1997). Magnesium might be required in the differentiation of the keratinocyte or barrier homeostasis.

Barrier homeostasis after damage to the barrier includes a series of biochemical phenomena such as an increase of lipid synthesis and lipid processing (Elias and Menon, 1991). Previous reports, however, suggest that the lipid metabolism started at least several hours after barrier disruption (Feingold and Elias, 2000). Thus, the fast acceleration of barrier repair by the negative electric potential might not be induced by lipogenesis but by a kind of biophysical

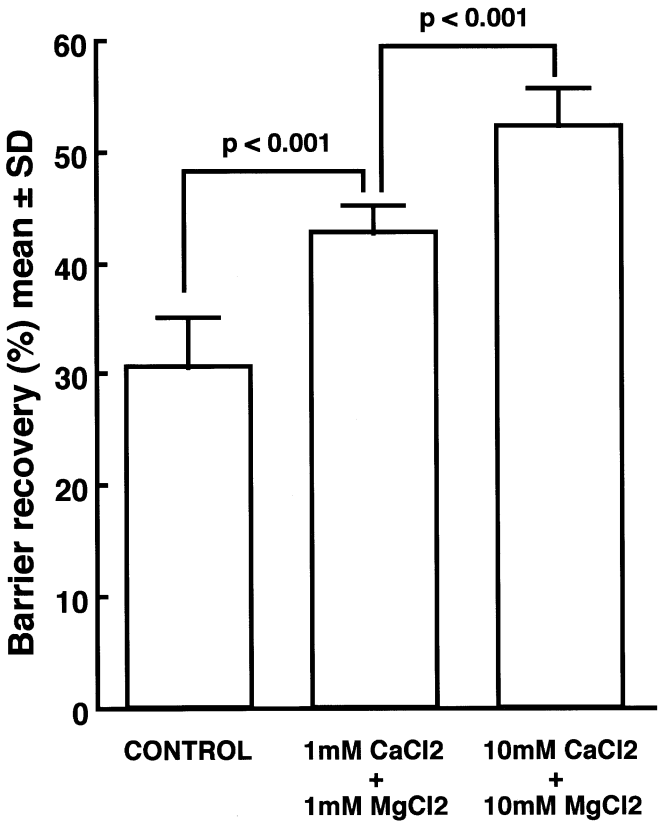

Figure 7. Topical application of a mixture of calcium chloride and magnesium chloride accelerated barrier recovery 1 h after barrier disruption by acetone treatment. The effect of the mixture was concentration-dependent (n = 8, each).

process. Ultrastructural studies suggested the acceleration of lamellar body secretion under the negative potential. Fusion of cell membrane and granules is a critical stage of exocytosis and the phase transition of the lipid membrane is strongly influenced by the ion concentration, especially calcium and magnesium (Ortiz *et al*, 1999). Electrostatic interaction also plays an important part in lipid membrane fusion (Montal, 1999). Phase transition of the lamellar bodies and keratinocyte cell membrane by alteration of the ionic or electrostatic field might be the cause of the acceleration of lamellar body secretion and barrier repair. Abraham *et al* (1987) demonstrated that calcium ion induced transformation of SC lipid liposomes into broad lamellar sheets, such as the intercellular lipid bilayer structure in the SC. We previously reported that alkyl chain conformation of the intercellular lipid molecules correlated with skin barrier function (Denda *et al*, 1994). Ionic and/or electrostatic alteration might also affect the intercellular lipid bilayer structure in the SC. Barrier recovery in the negative potential area was greater than that in the control area even 5 h after the end of the potential loading. This suggests that the acceleration of lamellar body secretion immediately after barrier disruption is an important stage for the barrier repair process as previously reported (Elias and Menon, 1991). The physicochemical features of the lipid phase in the upper epidermis under various ionic or electrostatic fields should be investigated.

The ion gradation in the epidermis might be important for cutaneous pathology. The ion profile has been reported to be altered in various skin diseases (Forslind *et al*, 1999). Abnormal calcium distribution was observed in psoriatic epidermis and atopic dermatitis. The distribution of zinc and iron was also altered in atopic skin. Ions might play an important part in the pathology of the skin. During the wound healing process, the distribution of magnesium and calcium in the wound fluid is altered, and may activate the cell migratory response (Grzesiak and Pierschbacher, 1995). Recent studies suggest that an abnormality in the calcium dynamics in epidermal keratinocytes is a crucial cause of cutaneous diseases. For example, mutation of the calcium pump of the keratinocyte is a cause of Darier's disease (Sakuntabhai *et al*, 1999). Karvonen *et al* (2000) demonstrated that psoriatic keratinocytes have an inborn error in calcium signaling. As the present study suggested, the external electric potential affects the ion localization in the epidermis. Potential loading might be a good strategy to improve cutaneous diseases caused by abnormal ion dynamics. Biophysical studies on the ion distribution in the epidermis of cutaneous diseases should be performed.

Acceleration of barrier repair after its damage could prevent inflammatory responses induced by barrier disruption (Denda *et al*, 1997; Ashida *et al*, 2001). The skin barrier homeostatic system of human skin is similar to that of hairless mouse (Elias and Menon, 1991). Acceleration of barrier repair by topical application of the same agents e.g., T-AMCHA or physiological lipid mixture, were observed in both hairless mice and human skin (Denda *et al*, 1997; Zettersten *et al*, 1997). Thus, a new strategy for improving barrier repair may regulate ion localization by external electric potentials in human skin. In the present study, we loaded both positive and negative potentials on the same mouse. Although the negative side showed acceleration of barrier repair, the positive side showed a delay of the repair. If one would try the electric potential load for critical usage, i.e., improvement of skin barrier function, the positive side should be grounded to avoid a side-effect of the positive potential. This strategy could result in novel therapeutic approaches to treat the cutaneous disorders caused by barrier damage or abnormal ion dynamics in keratinocytes.

In conclusion, the external negative electric potential induced epidermal lamellar bodies into the SC/SG interface and accelerated skin barrier recovery after barrier disruption. Localization of calcium and magnesium in the outermost part of the epidermis was also induced by the potential. Consequently, these results suggest that the external potential affects epidermal ion distribution and skin barrier homeostasis.

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