Biochemical and Biophysical Research Communications **272,** 134–137 (2000)
doi:10.1006/bbrc.2000.2739, available online at http://www.idealibrary.com on IDEAL®

Visual Imaging of Ion Distribution in Human Epidermis

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Received April 17, 2000

The distribution of calcium, magnesium, potassium, sodium, and hydrogen ions in the human epidermis was visualized by blotting to gel containing chemical indicators and the effects of skin barrier disruption were examined. In normal skin, both calcium and magnesium were localized with high concentration in the upper epidermis. EDTA blocked these imaging. The hydrogen ion was also high in the upper epidermis. Sodium did not show obvious gradation in the epidermis. The potassium concentration was the lowest in the upper epidermis. After the barrier disruption, the gradients of calcium, magnesium, and potassium disappeared while the pH gradation was not altered. Observation at a high magnification revealed lower calcium and sodium concentrations in the nucleus. The concentration of magnesium was slightly higher in the nucleus. The novel method of the present study could show the visual image of the ions in frozen tissue without further preparation. © 2000 Academic Press

Key Words: **calcium; magnesium; potassium; sodium; pH; stratum corneum; barrier; skin; keratinocyte.**

Studies suggest that ionic signals such as calcium and potassium play an important role on the homeostatic mechanism of the epidermal barrier function (1, 2). Thus, observation of these ions would provide us important information to understand epidermal homeostasis. However, image analysis of ions in the skin is technically difficult. Previously, the distribution of calcium and potassium has been studied by electron microscopic analysis after calcium precipitation (2) or by PIXE analysis (3, 4) of the skin. Although these gave us important quantitative information, some other important elements, such as hydrogen or magnesium, could not be observed by these methods because of their low atomic weight. Moreover, since these methods require dehydration or fixation of the sample without destroy the native chemical composition, they require complicated processes. Secondary ion MS (SIMS)-based imaging technique also can be used for observation of intercellular elements (5). But this method also requires a freeze-dry process. On the other hand, a variety of chemical indicators for each element have been used in the cell culture system (6) or even living tissue (7). Diffusion of metal ions in water is relatively slow (8). Prevention of diffusion of chemical indicators might allow us to observe the distribution of elements by these chemical probes in a tissue section. Freezing cells or small sections at low temperature using organic solvents may preserve a native distribution of diffusible ionic species (5). In the present study, we demonstrate a new method of visualization of magnesium, calcium, potassium, sodium, and hydrogen ions in skin frozen tissue of human skin. Further, modulation of the distribution pattern by tape stripping was detected.

MATERIALS AND METHODS

Calcium Green 1, Magnesium Green, PBFI and Sodium Green were purchased from Molecular Probes (Eugene, OR). Bromocresol Green was purchased from Wako, Osaka, Japan. Agarose was purchased from Sigma (St. Louis, MO).

Skin. Samples were obtained from the inner forearm of healthy males who gave their informed consent. The samples were biopsied from untreated skin and 30 min after tape stripping. To avoid the misobservation of artifacts, at least three samples were taken from one region. Tape stripping was carried out 20 times. At that time, most of stratum corneum removed but still some remained. Samples were immediately frozen in isopentane- filled metal jar which kept in liquid nitrogen to prevent artifactual redistributions (5). The frozen samples were kept at −80°C until sectioning.

Visualization of ions. Agarose gel (final 2%) contained each indicator was spread on the slide glass with 50μm thickness. For calcium observation, final concentration 10 μg/ml of calcium green 1 was mixed before the formation of the membrane. For magnesium, the final concentration 10 μg/ml magnesium green and 0.2 mM final concentration EGTA were mixed together. For potassium, the final concentration 10 μg/ml of PBFI was mixed. For sodium, the final concentration 10 μg/ml sodium green was mixed. In the case of hydrogen ion (pH) observation, first agarose gel membrane was formed and then 20 μl of 0.01% Bromocresol Green ethanol solution was spread over the gel membrane. A frozen section, 10 μm in thickness, was put on the gel membrane and within a couple of hours, the whole picture was taken. Within 12 h after the preparation, the clear images were disappeared. We used an Olympus microscope system (AH3-RFC, Olympus, Tokyo, Japan) for observation of the present study. For Calcium Green 1, Magnesium Green, and Sodium Green, the wavelength of the excitation light was 546 nm. For the potassium indicator, PDF1, the wavelength of the excitation light was 334, 365 nm. For each observation, at least five sections were observed to find common features.

0006-291X/00 $35.00

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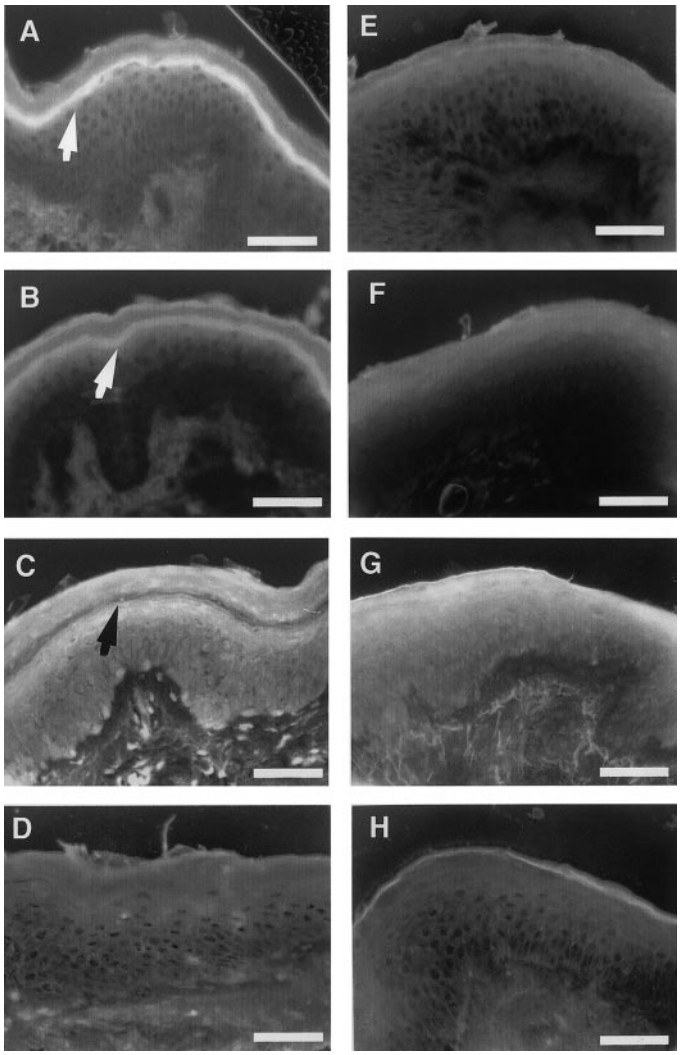

FIG. 1. Calcium localized in the epidermal granular layer in normal skin (A; white arrow). Thirty minutes after tape stripping, the gradation disappeared (E). Magnesium also showed the same tendency (B, white arrow; before tape stripping, F; after tape stripping). On the contrary, the concentration of potassium was higher in the spinous layer (C, black arrow) and after tape stripping, this gradation disappeared (G). On the other hand, sodium showed a homogeneous distribution around the whole epidermis (D), which was not affected by tape stripping (H). Bars, 50 μm.

RESULTS

Images of calcium, magnesium, potassium and sodium in the skin are shown in Fig. 1. These are the representatives of each observation. In normal skin, calcium was localized in the epidermal granular layer (Fig. 1A). Thirty minutes after tape stripping, this gradation disappeared (Fig. 1E). Magnesium also showed the same tendency (Figs. 1B and 1F). On the contrary, the concentration of potassium was the highest in the spinous layer and the lowest in the granular layer (Fig. 1C). This gradation also disappeared after tape stripping (Fig. 1G). Sodium showed a homogeneous distribution around the whole epidermis (Fig. 1G), which was not affected by tape stripping. To con-

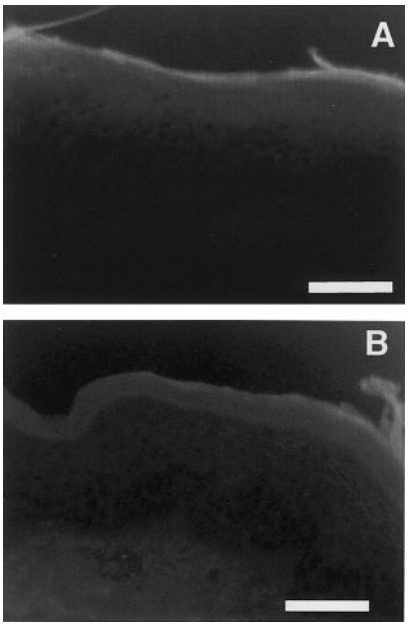

FIG. 2. EDTA absorbed the fluorescence of both calcium (A) and magnesium indicators (B). To confirm the results shown in Fig. 1, we also carried out the same experiment using a 50 mM of EDTA solution (Figs. 1C and 1F). EDTA absorbed the fluorescence of both indicators. This indicates that the images in Fig. 1 are due to each ion.

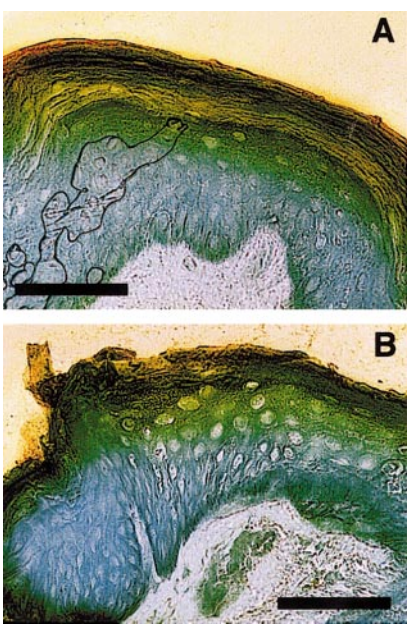

FIG. 3. The gradation of pH in the skin before and after tape stripping. In both cases, the stratum corneum and upper epidermis were more acidic (4.0–4.5 indicated by yellow-green). A deeper part of the epidermis and dermis were less acidic (higher than 5.6 indicated by blue-green). No obvious difference in pH gradation was observed after tape stripping. Bars, 50 μm.

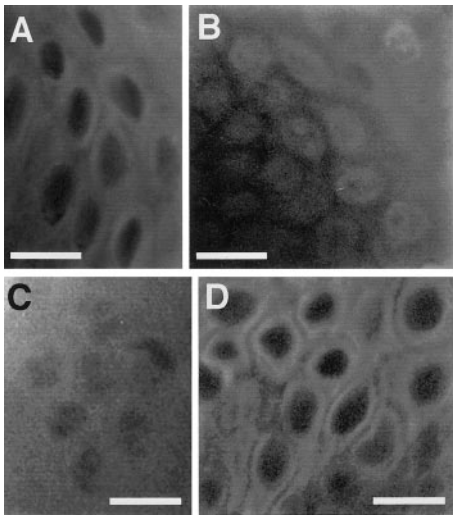

FIG. 4. The epidermal spinous layer after tape stripping at a higher magnification. The distribution of calcium (A) and sodium (D) showed heterogeneous pattern. In both cases, ions were absent in the nucleus. On the other hand, the magnesium concentration was slightly higher in the nucleus (B). Potassium did not show a clear distribution pattern (C). Bars, 10 μm.

firm these results, we also carried out experiments using calcium or magnesium indicators using a 50 mM EDTA solution (Figs. 2A and 2B). EDTA absorbed most of the fluorescence in the epidermis. This indicates that the gradation in Fig. 1 showed the distribution of each ion. The gradation of pH in the skin before and after tape stripping are shown in Fig. 3. In both cases, the stratum corneum and upper epidermis were more acidic (4.0–4.5 indicated by yellow-green). A deeper part of the epidermis and dermis were less acidic (higher than 5.6 indicated by blue-green). No obvious difference in pH gradation was observed after tape stripping. Pictures of the spinous layer of the epidermis at a high magnification after tape stripping are shown in Fig. 4. Calcium (Fig. 4A) and sodium (Fig. 4D) distribution showed an obvious pattern. In both cases, these ions were absent in the nucleus. On the other hand, after tape stripping, the magnesium concentration was slightly higher in the nucleus (Fig. 4B). Potassium did not show a clear distribution pattern (Fig. 4C).

DISCUSSION

Polymer gel, such as agarose or polyacrylamide, forms three-dimensional structure and prevents a water flow in side the structure (9). Thus, one can utilize them for electrophoresis or *in situ* zymography. In the present study, the diffusion of the chemical indicators might be prevented and the agarose membrane showed the images of the ion distribution.

Ionized calcium is the most common signal transaction element (10). Previously, the specific localization of calcium in the murine and human epidermis has been reported (2, 3). In the normal epidermis, the calcium concentration is higher in upper epidermis, i.e., granular layer and lower in the deeper epidermis, i.e., basal layer. Our present findings are in agreement with these reports. Mauro *et al.* reported (3) that this calcium gradient in the hairless mice epidermis disappeared immediately after barrier disruption. In our human study, the peak of calcium concentration in the epidermal granular layer also became broad. These results suggest that calcium play an important role in barrier homeostasis and/or signaling of barrier insults.

The present study showed that magnesium also formed a gradation in the epidermis like calcium. This result agrees to previous data (11, Fig. 2B). Moreover, this gradation also disappeared by barrier insults. The role of magnesium in the epidermis remains unknown. We previously demonstrated that application of magnesium after tape stripping accelerates barrier recovery (12). Magnesium is required for the activity of Rab-geranylgeranyl transferase which modifies Rab (13). After the modification, Rab plays an important role on exocytosis and endocytosis (14). For the 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 (15). Magnesium might be required in the differentiation of the keratinocyte or the barrier homeostasis.

Previous studies suggest a close relation between potassium and calcium (16). Both ions play an important role in the terminal differentiation of keratinocyte or stratum corneum barrier function. In our present study, potassium and calcium were localized comparably before and after barrier disruption. Warner *et al.* reported the lowest potassium concentration between the upper epidermis and the stratum corneum in human skin (17). And the disappearance of the potassium distribution in our present study was similar to the previous study of hairless mice (3). The relative localization of these ions might be important for epidermal homeostasis.

The mechanism of the quick movement of calcium, magnesium and potassium after the barrier insult remains unknown. There is an electric potential between the surface (negative) and bottom (positive) of the skin (18). This potential is erased immediately by barrier disruption and with slow recovery (19). Mauro *et al.* reported that the lost gradient of calcium recovered gradually after the barrier insults (3). Edelberg suggested that the skin surface potential reflected the sweat gland and the "epidermal generator" (19). The skin surface potential might be induced by the distribution of the ions. In this area, both biochemical and biophysical studies are needed.

Sodium did not show a gradation in the epidermis. This is in agreement with previous works (11, Fig. 4). The barrier insults did not affect the sodium ion dis-

tribution. Previous works demonstrated that topical application of sodium after the barrier disruption did not affect its repair response (1). These results suggest that sodium is not directly related to the cutaneous barrier homeostasis.

Localization in each cell was different between calcium and magnesium after barrier disruption. The concentration of calcium was low in the nucleus and relatively high in the cytosol. The concentrations of sodium in the cytosol was also higher than in the nucleus. On other hand, the magnesium concentration after tape stripping is slightly higher in the nucleus than in the cytosol. Magnesium ion has an ability to decrease the entropy of the water structure. Thus, magnesium plays a role to stabilize the nucleus protein structure (20). Magnesium might be important against environmental insults.

A lower pH in the upper epidermis would be important for barrier formation because lipid processing enzymes require it (21). Previously, application of basic buffer solution has been shown to delay the barrier recovery. The epidermis has a mechanism to keep the pH gradient for the barrier homeostasis (22). However, this gradation was not altered by barrier insults. The mechanism might be different from that of other divalent ions. Chapman and Walsh reported (23) that lamellar bodies (membrane-coating granules) are responsible for maintaining an acidic pH in the SC.

The ion profile has been reported to be altered in various skin diseases (4). 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 role 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 (24).

The novel method we presented here might need further methodological improvement to get accurate quantitative information; however, it would be useful to investigate the role of the ions in the skin or even other tissue.

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