Original Article

Study on the Epidermal Basement Membrane (2): Localization of Type VII Collagen Determined by Immunoscanning Electron Microscopy with Backscattered Electron Imaging

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Abstract: Localization of type VII collagen was investigated in the epidermal basement membrane. The interstitial surface of lamina densa was exposed by dithiothreitol treatment and immunohistochemistry was performed on this surface with anti-type VII collagen monoclonal antibody and the colloidal gold-conjugated secondary antibody. Colloidal gold particles were detected only on the anchoring fibrils by backscattered scanning electron microscopy. The fact that the type VII collagen was a component of anchoring fibrils was reconfirmed in the present study in the three-dimensional morphology.

Key words: type VII collagen, epidermal basement membrane

Introduction

The scanning electron microscopical observation on lamina densa has been performed only on the cellular surface¹². This is because the epidermal-dermal separation generally occurred at the lamina lucida of the epidermal basement membrane by the treatment with digestive enzymes¹. Dithiothreitol treatment is a rarely used technique for separating only the cellular surface became free and was subjected to the scanning electron microscopy (SEM). The connection between the lamina densa and the underlying collagen layer was not significantly compromised by these treatments. Dithiothreitol treatment is a rarely used technique for separating the epidermis from the dermis. After this treatment, the epidermis was mechanically peeled off, and at the same time anchoring fibrils were pulled off from the dermis⁴⁷. The dithiothreitol-separation is thought to be the only way to separate the lamina densa from the dermis, preserving the connection between the epidermis and lamina densa. Osawa and Nozaka⁷ first observed the interstitial surface of the lamina densa by SEM using the specimen prepared by dithiothreitol-separation. By their observations, it became clear that the lamina densa had a meshwork structure composed of fine fibrils, and anchoring fibrils projected from the meshwork to the interstitial space. The main component of these anchoring fibrils was revealed as type VII collagen by immunoelectron microscopic studies⁸⁻¹⁰. It is expected that the immunoscanning electron microscopic study of the dithiothreitol-separated basement membrane will confirm the three-dimensional localization of the components of the basement membrane, such as type VII collagen, on the interstitial side. Backscattered scanning electron imaging has the great advantage of being able to detect gold particles conjugated to the secondary antibody, because of the striking contrast between the gold particles and the background¹⁰⁻¹². In the present study, the immunostained specimens with colloidal gold-conjugated secondary antibody were observed using both secondary and backscattered scanning electron microscopy. By comparing these two kinds of electron micrographs the exact localization of type VII collagen was revealed.

Materials and Methods

Two-day-old Wistar rats (n=5) were used as materials in the present study. The skin on the back was cut out and incubated in 0.01 M dithiothreitol in phosphate-buffered saline (PBS) for one hour. After this treatment the epidermis was mechanically peeled off with a forceps into small pieces (n=20). The immunohistochemistry of the pieces of peeled epidermis was performed using the following reagents in sequence: (1) 0.05 M PBS containing 0.1% bovine serum albumin (BSA), 2 hr, (2) anti-type VII collagen monoclonal antibody (YLEM S.r.l.) diluted 1:10 in PBS-BSA, 2 hr, (3) washed three times, 20 minutes each in PBS-BSA, (4) 15 nm colloidal gold-conjugated goat anti-mouse IgG (Zymed Laboratories) diluted 1:10 in PBS-BSA, 2 hr, (5) washed three times, 30 min each, in PBS-BSA, and (6) washed in PBS. For the control, the first antibody was omitted. Then the pieces of peeled epidermis were fixed with 2% glutaraldehyde in a 0.1 M cacodylate buffer for 24 hr. A part of the specimens were processed for the transmission electron microscopy, i.e., post-fixed with 1% OsO₄ in a 0.1M cacodylate buffer (pH 7.4) for one hr, dehydrated through a graded series of ethanol, embedded in Epon 812, and observed with a Hitachi H-700H or a H-600A transmission electron mi
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croscope. This was done to confirm the separation of the epidermal basement membrane and the underlying dermis at the lamina fibroreticularis, exposing the interstitial surface of the lamina densa and to confirm the immunohistochemical reaction on the exposed surface. The rest of the specimens were post-fixed with 1% OsO₄ in a 0.1 M cacodylate buffer for 15 min and dehydrated through a series of ethanol. After the substitution from ethanol to 2-methyl-2-propanol, specimens were freeze-dried in an Eiko ID-2 freeze drier, mounted on SEM stubs, coated with osmium in an osmium plasma coater (Nippon Laser & Electron lab. MW-PC30), and observed under a Hitachi S-800 scanning electron microscope with 25 kV of acceleration voltage. Pairs of secondary electron images and the backscattered electron images were taken for the same fields of the specimens.

Results

The observation by the transmission electron microscopy showed that the epidermal-dermal separation occurred within the lamina fibroreticularis. Anchoring fibrils were pulled off from the dermis. Gold particles, which indicate the localization of type VII collagen, were seen only on the anchoring fibrils, but neither in the lamina densae nor in other locations (Fig. 1).

Fig. 1 Transmission electron micrograph showing the localization of type VII on anchoring fibrils with 15 nm colloidal gold particles. E, epidermis; LD, lamina densa; af, anchoring fibrils. Bar= 1μm.

Fig. 2a Secondary electron image of the undersurface of dithiothreitol-separated epidermal basal lamina. Numerous anchoring fibrils project from lamina densa to the interstitial side and they are connected at the distal ends (arrows).

2b Backscattered electron image of the same field as Fig 2a. Gold particles are seen on the indistinct anchoring fibrils with striking contrast (arrows). Bar= 0.1μm.
No gold particles were seen on the control specimen (data not shown). Scanning electron microscopic observation with secondary electrons revealed the three-dimensional morphology of the anchoring fibrils. They projected from the lamina densa and were connected with each other at the distal ends (Fig. 2a).

Backscattered electron images showed not only the strikingly contrasting gold particles, but also indistinct anchoring fibrils. The positions of the gold particles were identified by the comparison between the secondary and the backscattered electron images in the same fields. No gold particles were seen in the lamina densa, but were found only on the anchoring fibrils. Gold particles sometimes made clusters in some places (Fig. 2b). No gold particles were seen on the control specimen (data not shown).

Discussion

By the comparison between the secondary and the backscattered electron images in the same fields, it became clear that the gold particles were only found on the anchoring fibrils. This localization coincided with the transmission electron microscopic observations. In the present study, it was reconfirmed that the anchoring fibrils contain type VII collagen as a component, and we could show this fact in the three-dimensional morphology. Backscattered electron imaging showed the great advantage of the colloidal gold-conjugated antibodies because of the striking contrast in the image. In the present study the specimens were prepared using osmium fixation and coating. Both of the treatments were needed for the stability of the scanning electron images; however, it was supposed that osmium fixation and coating would decrease the contrast of the backscattered images of the gold particles. In fact, despite the osmium fixation and osmium coating of the specimens, the gold particles were observed with striking contrast. In the present study, osmium fixation for a short time and thin osmium coating of the specimens, and at the same time we could obtain stability of the scanning electron images by these treatments.

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