Methods in Molecular Biology (2005) 289:223-226.

Immunoelectron microscopic analysis of cornified cell envelopes and antigen retrieval

Ishida-Yamamoto, Akemi

Immunoelectron microscopic analysis of cornified cell envelopes and antigen retrieval

Running head: Immuno-EM of cornified cell envelopes

Akemi Ishida-Yamamoto, MD, PhD

Department of Dermatology, Asahikawa Medical College, Asahikawa, Japan

Send correspondence to: Dr. Akemi Ishida-Yamamoto, Department of Dermatology, Asahikawa Medical College, Asahikawa, 078-8510 Japan. Phone: 81-166-68-2523: Fax Number; 81-166-68-2529; E-mail: akemi@asahikawa-med.ac.jp

Abstract

In this section, post-embedding immunoelectron microscopy methods for studies of cornified cell envelopes are provided. As the material, human epidermal tissue samples are used. The samples are cryo-fixed without chemical fixation, freeze-substituted at a low temperature, and embedded in Lowicryl K11M resin. For immunostaining, colloidal gold conjugated secondary antibodies are used. Methods for retrieval of masked epitopes are also described.

Key Words

immunoelectron microscopy, post-embedding method, cornified cell envelopes, keratinocytes, involucrin, loricrin, epitope masking

1. Introduction

The cornified cell envelope (CE) is a highly insoluble structure formed beneath the plasma membrane of differentiating keratinocytes (1). It consists of various cross-linked precursor proteins, including involucrin and loricrin. During the last several years, we have begun to understand the normal assembly process of CE and its alteration in skin disorders. In this chapter, protocols to study the localization of CE proteins using post-embedding immunoelectron microscopy will be described in detail (2). Since some of the epitopes of CE proteins are masked during the assembly of CE or tissue processing, methods for antigen retrieval will also be provided (3, 4). All of the protocols are developed for the use with human skin.

2. Materials

2.1. Antibodies for immunoelectron microscopy

1. Rabbit polyclonal antibody against loricrin (AF62) (BAbCO, Richmond, CA, cat no.

PRB-145P).

2. Mouse monoclonal antibody against involucrin (SY5) (NeoMarkers, Fremount, CA, cat no. MS-126-P).

3. Rabbit polyclonal antibody against involucrin (BT-601) (Biomedical Technologies, Stoughton, MA, cat no. BT-601).

2.2. Reagents

2.2.1. Preparation of resin-embedded tissue samples

1. Lowicryl K11M (TAAB Laboratories, UK, cat no. L016).

2. Nickel 150-grid mesh (VECO®Plain Nickel Grids, Ernest F. Fullam, Inc. NY, cat no. 26121).

3. Formvar: 3% Formvar Soluction (LADD Research Industries, cat no. 12065), dilute at 0.3% in 1,2-dichloroethane. Store at room temperature up to 1 mo.

2.2.2. Immunoelectron microscopy

1. Fish Gelatin (Amersham Biosciences, Buckinghamshire, UK, cat no. RPN416).

2. Pre-incubation buffer: 1% bovine serum albumin (BSA), 5% normal goat serum (NGS), 0.1% fish gelatin, 0.02% sodium azid / phosphate buffered saline pH7.4 (PBS).

3. 1st incubation buffer: 1% BSA, 1% NGS, 0.1% fish gelatin, 0.02% sodium azide /PBS.

4. Tris-HCl buffered saline pH 8.2 (TBS): 0.242g Tris (tris-hydroxmethyl-aminomethane), 0.13g NaN_3 (sodium azide), 0.9g NaCl; Adjust pH with 0.1N HCl to 8.2. Add dH_2O to final total volume of 100 mL.

5. 2nd incubation buffer: 1% BSA, 1% NGS, 0.1% fish gelatin, in TBS.

6. 5 nm gold-conjugated goat anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, UK, cat no. RPN420).

7. 10 nm gold-conjugated goat anti-rabbit IgG (Amersham Biosciences, cat no. RPN421).

8. 5 nm gold-conjugated goat anti-mouse IgG (Amersham Biosciences, cat no. RPN424).

9. 10 nm gold-conjugated goat anti-mouse IgG (Amersham Biosciences, cat no. RPN425).

2.2.3. Antigen retrival

1. Proteinase K (DAKO, Carpinteria, CA, cat no. S3020): store at 50 mM in Tris-HCl buffer, pH7.4 at –30°C, up to 3 mo.

2. Trypsin (Difco, Detroit, MI, cat no. 0152-13-1): Stored at 0.25% in PBS at –30°C.

2.3. Equipments and vials for cryo-fixation and freeze-substitution

1. Reichert KF80 cryo-fixation apparatus (Leica, Wien, Austria).

2. Cryogenic vial (Nalge Company, Rochester, NY. cat no. 5000-0020).

3. Reichert AFS automatic freeze-substitution system (Leica)

3. Methods

3.1. Tissue preparation for post-embedding immunoelectron microscopy

This section describes the method to prepare tissue samples used for post-embedding immunoelectron microscopy. By using cryo-fixation, all components of biological specimens are immobilized without the use of any of chemical fixation procedures and the damaging effects on sensitive and labile antigenic sites can be avoided. The ice in the specimen is then dissolved and replaced by an organic solvent at low temperature by a freeze-substitution method. This enables us to avoid denaturation of proteins that can occur during the curing of resins at higher temperatures.

3.1.1. Cryo-fixation

1. Immerse skin tissue samples immediately after a biopsy or surgical operation into ice-cold 15% glycerol/PBS for 1h. Samples should be cut into small pieces less than 1 mm^3.

2. Mount a sample on a bare copper grid (VECO) held with forceps.

3. Plunge it rapidly into liquid propane at -190°C using a cryo-fixation apparatus.

4. Store in a cryogenic vial in liquid nitrogen.

3.1.2. Freeze-substitution

1. Transfer cryo-fixed tissue samples to plastic capsules with mesh bottom containing substitution liquid (methanol) placed in the substitution chamber of a automatic freeze-substitution system at -80°C.

2. Continue substitution for 44 h, exchanging substitution liquid twice a day.

3.1.3. Low temperature embedding

1. Raise the temperature 5°C/h up to -60°C and start infiltration with embedding medium in the dark.

2. Prepare fresh Lowicry K11M.

3. Exchange the substitution solution against embedding medium by increasing concentration of Lowicryl in methanol as follows.

a) Lowicryl K11M/methanol: 1:2 (vol:vol) 1 h

b) 1:1 1h

c) 2:1 1h

d) Pure Lowicryl K11M 1 h

e) Pure Lowcryl K11M overnight

f) Embed in fresh Lowicryl K11M filled in plastic molds.

4. Initiate polymerization under ultraviolet radiation for 48 h at -60°C, raise the

temperature 5°C/h up to 20°C and continue polymerization for further 48 h.

3.2. Immunostaining

This section describes the section preparation methods and immunostaining methods for post-embedding immunoelectron microscopy.

3.2.1. Sectioning procedure

1. Remove the specimen embedded in Lowicryl K11M from plastic molds.

2. Cut 100 nm-thick sections using a ultra-microtome and collect them on Formvar-coated nickel mesh (see Note 1).

3. Blot on filter paper and air dry.

3.2.2. Immunostaining procedure

Immunostaining is done on drops of solutions placed on a piece of laboratory film. (Parafilm, American Natinal Can, Chicago, IL). (For negative control studies and double labeling, see Notes 2 and 3, respectively)

1. Incubate the specimen-mounted grids on a drop of pre-incubation buffer for 15 min at room temperature.

2. Transfer to a drop of one of the primary antibody solutions (anti-loricrin antibody AF62, 1:500 dilution, anti-involucrin antibody SY5, 1:100 dilution, anti-involucrin antibody BT-601, 1:5 dilution) diluted in 1st incubation buffer and incubate for 1 h at 37°C.

3. Place on drops of 1st incubation buffer twice for 5 min at room temperature.

4. Place on drops of 2nd incubation buffer twice for 5min at room temperature.

5. Incubate on a drop of appropriate colloidal gold-conjugated secondary sera (gold particle size 5- or 10-nm, 1:10 dilution in 2nd incubation buffer) for 1 h at 37°C.

6. Place on drops of 2nd incubation buffer twice for 5 min at room temperature.

7. Place on drops of dH_2O twice for 5 min at room temperature and rinse gently in running dH_2O.

8. Blot on filter paper and air-dry on a piece of laboratory film.

9. The sections were then contrasted with 1.5% uranyl acetate in methanol for 3 min.

10. Rinse in 50% methanol/ dH_2O.

11. Rinse in dH_2O.

12. Blot on filter paper and air-dry on a piece of laboratory film.

3.3. Antigen retrieval

Incubate ultrathin sections of Lowicryl K11M embedded skin tissue with either 0.4 mg/ml proteinase K in Tris-HCl buffer pH 7.4 for 30 s to 5 min at room temperature or with 0.25% trypsin in PBS pH 7.4 for 15 min to 60 min at 37 °C.

Rinse with the buffer (Tris-HCl buffer pH 7.4 and PBS pH 7.4, respectively) and immunostain as described above

4. Notes

1. Lowicryl K11M is a hydrophilic resin. Therefore, precautions should be taken to ensure that the block face does not become wet during sectioning. This is best accomplished by sectioning with a low water level in the knife-trough.

2. Negative controls included incubation in the presence of the secondary antibody alone and with primary antibodies against antigens that are not expected to be present in the epidermis.

3. For double labeling, stain with one primary antibody and label with 5- gold-conjugated secondary antibodies followed by staining with another primary antibody raised in a different animal and labeling with 10- nm gold conjugated secondary antibodies.

Acknowledgment

Development of the protocols described above was supported by grants from the ministry of education, culture, sports, science and technology and the ministry of health, labor and welfare of Japan.

References

1. Ishida-Yamamoto, A., Iizuka, H. (1998) Structural organization of cornified cell envelopes and alterations in inherited skin disorders. Exp Dermatol 7, 1-10.

2. Ishida-Yamamoto, A., Eady R.A.J., Watt, F. M., Roop, D. R., Hohl, D., Iizuka, H. (1996) Immunoelectron microscopic analysis of cornified cell envelope formation in normal and psoriatic epidermis. J Histochem Cytochem 44, 167-175.

3. Ishida-Yamamoto, A., Tanaka, H., Nakane, H., Takahashi, H., Iizuka, H. (1999) Antigen retrieval of loricrin epitopes at desmosomal areas of cornified cell envelopes: an immunoelectron microscopic analysis. Exp Dermatol 8, 402-406.

4. Ishida-Yamamoto, A., Kato, H., Kiyama, H., Armstrong, D.K.B., Munro, C.S., Eady, R.A.J., Nakamura, S., Kinouchi, M., Takahashi, H., Iizuka, H. (2000) Mutant loricrin is not crosslinked into the cornified cell envelope but is translocated into the nucleus in loricrin keratoderma. J Invest Dermatol 115, 1088-1094