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Original Article

Localization of ABCA12 from Golgi apparatus to lamellar granules in human upper epidermal keratinocytes

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Abstract: ABCA12 is an ATP-binding cassette transporter and is thought to act as a transmembrane lipid transporter. We reported that deleterious ABCA12 mutations cause a disturbance in lamellar granule (LG) lipid transport in the epidermal granular layer keratinocytes, resulting in harlequin ichthyosis, a severe genodermatosis. Detailed localization of ABCA12 in comparison with glucosylceramide and Golgi apparatus markers were studied in order to obtain clues to clarify the function(s) of ABCA12 in human skin. We performed double-labelling immunofluorescent staining using antibodies against ABCA12, glucosylceramide and two Golgi apparatus markers (TGN46 and GM130) in normal human skin and cultured keratinocytes. Immunogold electron microscopy for *ABCA12* and glucosylceramide was studied on postembedding and cryoultrathin sections of normal human skin. Confocal laser scanning microscopy demonstrated that ABCA12 and glucosylceramide co-localized in the granular layer keratinocytes as well as in keratinocytes cultured in high Ca^{2+} conditions through the Golgi apparatus to the cell periphery. Postembedding immunogold electron microscopy revealed that both ABCA12 and glucosylceramide labellings were associated with the LG of the uppermost granular layer keratinocytes. Using cryoultramicrotomy, lamellar structures in the LG were more clearly observed, and ultrastructural localization of *ABCA12* and glucosylceramide was better demonstrated to LG in the uppermost granular layer cells. These results indicate that *ABCA12* plays an important role in lipid transport from the Golgi apparatus to LG in human granular layer keratinocytes.

Key words: ATP – keratinization – lipid barrier – secretion – trafficking

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Introduction

The ATP-binding cassette (ABC) transporter superfamily is one of the largest gene families, encoding highly conserved proteins involved in energy-dependent transport of a variety of substrates across membranes, including ions, amino acids, peptides, carbohydrates, and lipids (1–4). The 48 currently known ABC genes are classified into seven subfamilies, based on sequence homology and organization of their nucleotide-binding folds. ABC genes are widely dispersed throughout the eukaryotic genome and are highly conserved between species (5). The ABCA subfamily, of which the ABCA12 gene is a member, comprises 12 full transporters and one pseudogene (*ABCA11*) and is thought to work in lipid transport (5). Among the several subclasses of ABC transporters, the ABCA subclass (6) has received considerable attention because of four ABCA genes in the subfamily: *ABCA1*, *ABCA3*, *ABCA4* and *ABCA12* that have been implicated in the development of genetic diseases affecting lipid transport (7,8). ABCA1 is a defective protein underlying Tangier disease (MIM 205400), familial hypoalphalipoproteinemia (MIM 604091) and premature atherosclerosis (9–11). *ABCA4* mutations underlie Stargardt disease (MIM 248200), some forms of autosomal recessive retinitis pigmentosa (MIM 601718) and autosomal recessive cone–rod dystrophy (MIM 604116) and macular degeneration (MIM 153800) (11–13). Cholesterol/phospholipids and protonated *N*-retinylidene phosphatidylethanolamine were suggested to be substrates for *ABCA1* and *ABCA4*, respectively (14–17). *ABCA1* and *ABCA7* were reported to be expressed in the epidermal keratinocytes (18,19).

Extracellular lipid including ceramide is thought to be essential for skin barrier function (20). Mutations in the ABC transporter A12 gene (*ABCA12*) were reported to underlie the devastating phenotype seen in Harlequin ichthyosis (HI) patients (21,22), the most severe keratinization disorder thus far known. *ABCA12* mutations

Abbreviations: ABC, ATP-binding cassette; ABCA12, ABC transporter A12; LG, lamellar granule.

underlying HI are thought to lead to major disruptive defects in ABCA12 lipid transporter function resulting in the HI phenotype (21). Since 2005, a number of HI patients with ABCA12 mutations have been reported in the literature (23–28). We reported previously that ABCA12 is localized in lamellar granules (LG) in the granular layer keratinocytes and might work in the lipid transport via LG to form the intercellular lipid layers in the stratum corneum (21). However, the detailed pathomechanisms of abnormal lipid accumulation in the keratinocyte cytoplasm and defective lipid secretion from keratinocytes has not been completely clarified in HI. The aim of the present study was to clarify the precise localization sites of ABCA12 in epidermal keratinocytes *in vivo* and *in vitro* in comparison with other keratinization-associated molecules and Golgi apparatus-associated molecules, and to have clues to further characterize the detailed function of ABCA12 in keratinocyte lipid transport. We have analyzed the epidermal localization of ABCA12 in comparison with the localization of Golgi apparatus markers and LG-associated proteins together with transglutaminase 1, because LG are thought to be a part of continuous tubular network originated from Golgi apparatus to the cell membrane. In this point of view, we employed antibodies to well-established marker molecules of each part of Golgi apparatus-LG-cell membrane network, i.e. GM130 antibody, anti-TGN-46 antibody and anti-transglutaminase 1 antibody (B.C1) as markers for *cis*-Golgi, *trans*-Golgi and cell membrane, respectively. Our results showed that ABCA12 localized throughout the entire Golgi apparatus to LG at the cell periphery mainly in the granular layer keratinocytes. These results suggest that ABCA12 works in lipid transport from Golgi apparatus to LG in the granular layer cells.

Materials and methods

Antibodies

Polyclonal anti-ABCA12 antiserum was raised in rabbit (21). The other primary antibodies used in the present study were mouse monoclonal anti-glucosylceramide antibody (Alexis Biochemicals, San Diego, CA, USA) for immunofluorescent labelling, rabbit polyclonal anti-glucosylceramide antibody (Glycobiotech, Kuekel, Germany) for immunoelectron microscopic labelling, sheep polyclonal anti-TGN-46 antibody (Serotec Inc., Oxford, UK), anti-transglutaminase 1 antibody, B.C1 (Biomedical Technologies Inc., Stoughton, MA, USA) and GM130 (BD Biosciences, San Jose, CA, USA).

Cell culture

Normal human keratinocytes were purchased from Lonza Walkersville Inc., (Walkersville, MD, USA) and were grown in defined keratinocyte serum-free medium (SFM) (Invitrogen, San Diego, CA, USA). All the cells were maintained at 37°C in a 5% CO_2 atmosphere.

In order to induce keratinization, both cells were cultured with high-concentration calcium medium (Ca^{2+} = 1.2 mM) for 48 h.

Immunofluorescent labelling

Immunofluorescent labelling was performed as previously described (29). Briefly, 5-μm-thick sections of patients' fresh unfixed skin samples and control normal skin samples cut using a cryostat or keratinocytes cultured in the chamber slides were prepared for immunolabelling. The sections and cells were incubated in primary antibody solution for 30 min at 37°C. Antibody dilutions were as follows: 1/10 for anti-glucosylceramide antibody, 1/4 for anti-TGN-46 antibody, 1/10 for anti-transglutaminase 1 antibody B.C1 and 1/10 for GM130.

The sections were then incubated in fluorescein isothiocyanate (FITC)-conjugated to rabbit anti-mouse immunoglobulins or goat anti-rabbit immunoglobulins diluted 1 : 50 (Jackson ImmunoResearch, Baltimore Pike, USA) for 30 min at 37°C, followed by 10 μg/ml of propidium iodide (Sigma Chemical Co., St. Louis, MO, USA) nuclear counterstain for 10 min. For double labelling, FITC-conjugated and tetramethylrhodamine-isothiocyanate (TRITC)-conjugated secondary antibodies were used to label deposits of rabbit polyclonal anti-*ABCA12* antiserum and mouse monoclonal antibodies, respectively. Nuclear staining was performed using TO-PRO-3 (Invitrogen, Carlsbad, CA, USA) in the double-labelled sections. The sections were extensively washed with phosphate-buffered saline (PBS) between incubations. The stained sections were then mounted with a cover slip in 50% glycerol-based mounting medium and observed by a confocal laser scanning microscope.

Postembedding immunogold electron microscopy using cryofixed, cryosubstituted samples

Normal human skin samples were obtained from surgical operations of the benign subcutaneous skin tumors under fully informed consent, and were processed for postembedding immunoelectron microscopy as previously described (30). Cyrofixed, cryosubstituted samples were embedded in Lowicryl K11M resin. Ultrathin sections were cut and incubated with rabbit polyclonal anti-*ABCA12* antisera or rabbit polyclonal anti-glucosylceramide antibody, a secondary linker antibody and a 5-nm gold-conjugated antibody for immunogold labelling.

Ultrathin cryosections (0.1–0.2-μm thick)

Ultrathin cryosections of normal human skin were obtained basically according to a method described by Tokuyasu in 1980 (31), with some modifications (32). Fresh normal human skin obtained at surgery was cut into

small pieces (<1 mm^3), prefixed with 2% paraformaldehyde at room temperature for 2 h, and with 100 mM glycine in Dulbecco's PBS at room temperature for 2 h and with 2.3 M sucrose in phosphate buffer at 4°C for 2 days. The samples were mounted on pins, rapidly frozen by liquid propane plunging (at −196°C), and stored in liquid nitrogen until required. Semi-thin sections were cut from the surface of the samples at −80 to −100°C using an Ultracut-S ultramicrotome equipped with a FCS cryosystem (Reichert-Jung, Vienna, Austria). Then, ultrathin sections (0.2-μm thick) were cut, transferred to albumin-coated glass slides using a platinum loop containing a droplet of 2.3 M sucrose/PBS, and processed as substrate for immunofluorescence labelling. For immunogold electron microscopy, ultrathin sections (0.1-μm thick) were also cut from a block, transferred to Formvar-coated nickel grids and processed for immunolabelling.

Results

Immunofluorescent double labelling revealed *ABCA12* and glucosylceramide co-localized within the granular layer keratinocytes

In normal human epidermis, ABCA12 is expressed in the entire epidermis, mainly in the upper spinous and granular layers (Fig. 1b,e,h,k). With non-specific background staining in the basal layer cells, the ABCA12 immunoreactivity seemed to be stronger than the true expression level. Glucosylceramide, a major lipid component of LG (33–35) was seen in the granular layer in the epidermis (Fig. 1a). TGase1, a membrane-bound enzyme essential for cornified cell envelope formation, was expressed at the cell periphery of the upper spinous and granular layer cells (Fig. 1d). TGN46, a trans-Golgi marker, was seen in the cytoplasm of all the epidermal cells (Fig. 1g). Perinuclear small dots staining of GM130, a *cis*-Golgi marker, was seen in the cytoplasm of keratinocytes in all epidermal layers (Fig. 1j). Immunofluorescent double labelling revealed that the majority of ABCA12 co-localized with glucosylceramide in the cytoplasm within the upper spinous and granular cells (Fig. 1c). ABCA12 seemed to partly co-localize with TGase1 (Fig. 1f). ABCA12 and TGN46 showed no apparent overlapping staining in the epidermis *in vivo* and only TGN46 expression was seen in the spinous layer cells (Fig. 1i). GM130 immunolabelling was seen mainly in the lower epidermis and no apparent co-localization of GM130 and ABCA12 was observed *in vivo* (Fig. 1l).

In cultured keratinocytes, ABCA12 is distributed from Golgi apparatus to the cell periphery

In normal human keratinocytes cultured under high Ca^{2+} conditions, the majority of ABCA12 and glucosylceramide co-localized in the cytoplasm (Fig. 2a–c). ABCA12 and

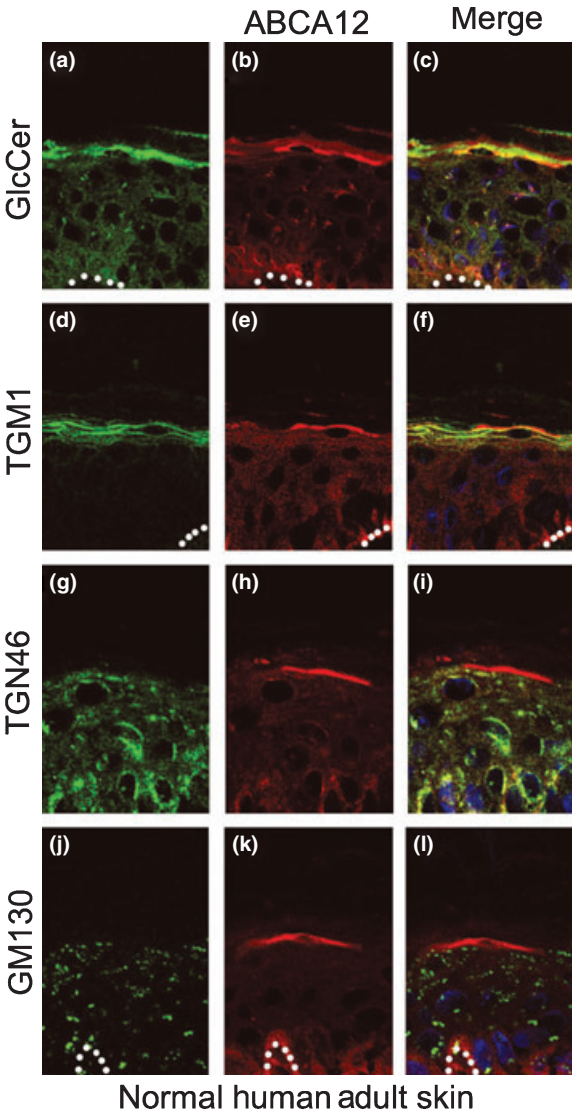

Figure 1. Double immunolabelling for ABCA12 and other molecules using conventional cryostat sections of normal human skin. (a–c) Double immunolabelling with anti-glucosylceramide (GlcCer) antibody and anti-ABCA12 antibody. GlcCer and ABCA12 immunolabelling overlapped in the cytoplasm of granular layer keratinocytes (c). (d–f) Double immunolabelling with anti-TGase1 antibody and anti-ABCA12 antibody. Both TGase1 and ABCA12 immunostaining were observed mainly in the granular layer cells and they partly overlapped (f). (g–i) Double immunolabelling with anti-TGN46 antibody and anti-ABCA12 antibody. TGN46 was detected in the cytoplasm of the spinous and granular layer cells (g,h). TGN46 and ABCA12 immunostaining showed no apparent overlap (i). (j–l) Double immunolabelling with anti-GM130 antibody and anti-ABCA12 antibody. Small, punctuate cytoplasmic staining was observed in the spinous and granular layer cells with anti-GM130 antibody (j). GM130 immunostaining did not overlap apparently with anti-ABCA12 antibody staining in the epidermis (l). ABCA12, red (TRITC); GlcCer, TGase1, TGN46, GM130, green (fluorescein isothiocyanate); nuclear stain, blue (TO-PRO-3). White dots, dermoepidermal junction. Magnification ×40.

TGase1 failed to co-localize. TGase1 staining showed a peripheral plasma membrane-associated pattern at the cell periphery (Fig. 2d–f). *ABCA12* staining was proximal to the TGase1 staining.

ABCA12 and TGN46 co-localized in part in the cytoplasm (Fig. 2g–i). GM130 staining was seen in the perinuclear area whereas the majority of GM130 was co-localized with ABCA12 around the perinuclear cytoplasm, although only ABCA12 staining extended to the cell periphery (Fig. 2j–l).

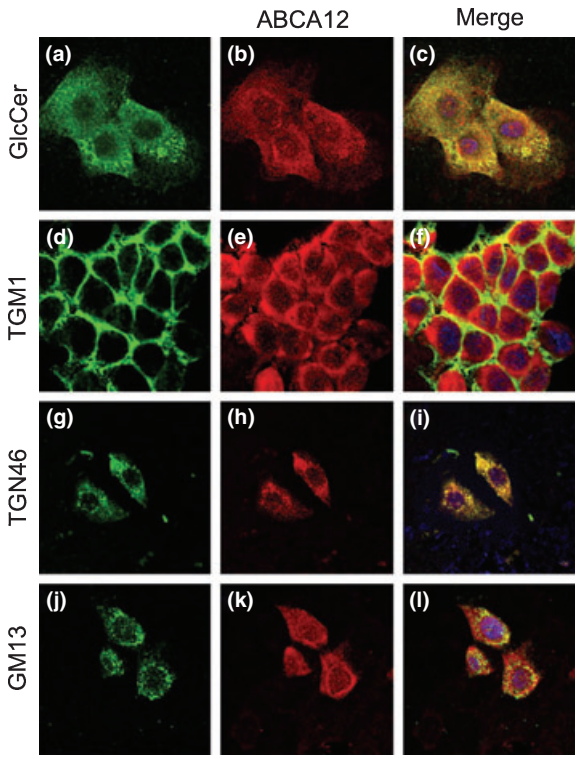

Figure 2. Double immunolabelling for ABCA12 and other molecules in normal cultured human epidermal keratinocytes. (a–c) Double immunolabelling with anti-glucosylceramide (GlcCer) antibody and anti-ABCA12 antibody. Both GlcCer immunostaining and ABCA12 immunolabelling were diffusely observed in the cultured keratinocyte cytoplasm (a,b). GlcCer and ABCA12 immunolabelling almost completely overlapped in the cytoplasm (c). (d–f) Double immunolabelling with anti-TGase1 antibody and anti-ABCA12 antibody. TGase1 seemed to be restricted to the cell membrane (d) and ABCA12 was observed diffusely within the cytoplasm (e) of the cultured keratinocytes. The immunostaining rarely overlapped (f). (g–i) Double immunolabelling with anti-TGN46 antibody and anti-*ABCA12* antibody. TGN46 was detected in the cultured keratinocyte cytoplasm (g). ABCA12 was rather diffusely distributed in the cytoplasm (h). Most TGN46 co-localized with ABCA12 (yellow colour) (i). (j–l) Double immunolabelling with anti-GM130 antibody and anti-ABCA12 antibody. GM130 was detected in the cultured keratinocyte cytoplasm, mainly in the perinuclear area (j). Most of GM130 co-localized with ABCA12 (yellow colour) in the perinuclear area, but at the cell periphery only ABCA12 immunostaining was observed without GM130 labelling (l). ABCA12, red (TRITC); GlcCer, TGase1, TGN46, GM130, green (fluorescein isothiocyanate); nuclear stain, blue (TO-PRO-3). Magnification ×60.

Postembedding immunoelectron microscopic observation revealed that both *ABCA12* and glucosylceramide localized to LG

Postembedding immunoelectron microscopy revealed that both ABCA12 and glucosylceramide were observed in the LG of the uppermost granular layer keratinocytes (Fig. 3). In the granular layer cells, there was no apparently labelled

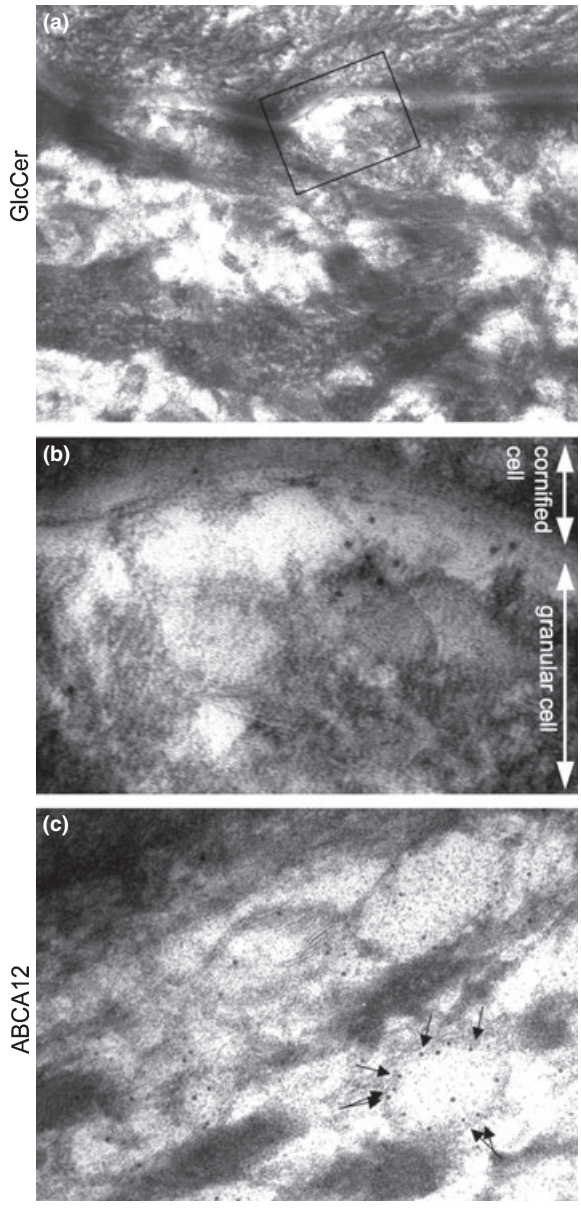

Figure 3. Cryosubstitution, cryofixation postembedding immunoelectron microscopy with anti-ABCA12 antibody and anti-glucosylceramide antibody. The apical border of the granular layer cell was observed (a). 5-nm gold labellings for glucosylceramide (b) and ABCA12 (c) were observed in the lamellar granules (LG) in the cytoplasm of the granular layer cells. ABCA12 labelling were seen on the LG (c, arrows). Magnification ×20 000 (a), ×1 00 000 (b), ×50 000 (c).

structure other than LG. Glucosylceramide was secreted from the LG to the intercellular space in the stratum corneum.

Cryoultramicrotomy

Ultrathin cryosections demonstrated highly preserved, easy to visualize plasma membrane ultrastructure of LG and intercellular lipid layers. In addition, immunofluorescence labelling on ultrathin cryosections clearly revealed a localization of ABCA12 and glucosylceramide. Using immunofluorescence labelling at the light microscopic level, ABCA12 and glucosylceramide staining almost completely overlapped within the granular layer keratinocytes (Fig. 4). By immunoelectron microscopy using ultrathin cryosections, glucosylceramide labelling was seen with the lamellar structures in the LG (Fig. 5a,b). ABCA12 immunogold labelling was observed on or close to the membrane surrounding LG in the uppermost granular layer cells (Fig. 5c,d).

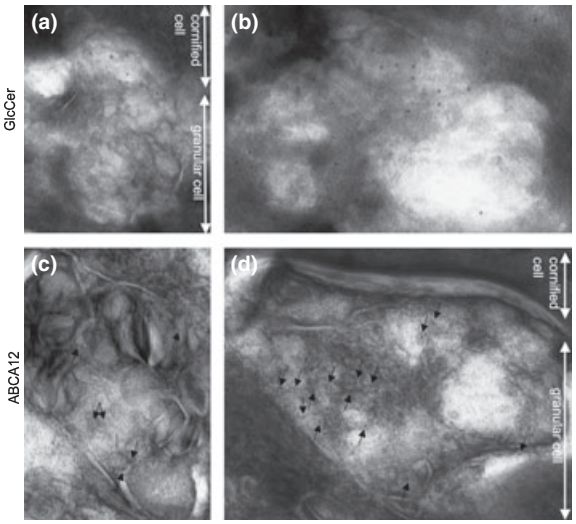

Figure 5. Cryoultramicrotomy for ABCA12 and glucosylceramide. Immunogold electron microscopic observation on ultrathin cryosections of normal human skin showed an apparent glucosylceramide (5-nm gold particles) labelling of the lamellar structure inside the lamellar granules (LG) (a,b). ABCA12 (5-nm gold particles) localized to LG containing lamellar structures (c,d). Magnification ×50 000 (a,b), ×30 000 (c,d).

Discussion

ABCA12 belongs to a large superfamily of ABC transporters, which bind ATP in the transport of various molecules across membranes (2–4). The ABCA subfamily is thought to act as lipid transporters (5).

ABCA3 was thought to work in lipid transport in type II alveolar cells in alveolar surfactant formation (36) and loss of function mutations in ABCA3 lead to fatal surfactant deficiency in the neonate (37). Finally, defects in *ABCA12* were identified as the cause of HI and lamellar ichthyosis (21,22,38).

ABCA2, *ABCA3* and *ABCA7* mRNA levels were reported to be upregulated after sustained cholesterol influx (39,40), suggesting that ABCA transporters are involved in transmembrane transport of endogenous lipids (15). From these facts, transporters in the ABCA subfamily were thought to be involved in transmembrane transport of endogenous lipids (14,16,17).

In the present study, we have demonstrated that the majority of ABCA12 was distributed in the granular layer cells associated with glucosylceramide. Double-labelling immunofluorescence staining in cultured keratinocytes clearly indicated that ABCA12 was localized from Golgi apparatus (co-localized with *cis*-Golgi marker GM130 and *trans*-Golgi marker TGN-46) to cell periphery (close to the plasma membrane stained with transglutaminase 1). In the epidermis *in vivo*, GM130 was expressed equally from the basal cells to the granular layer cells, although *ABCA12* was expressed mainly in the granular layer cells. Thus, co-localization of ABCA12 and GM130 was seen only in the limited area in the granular layers of the epidermis *in vivo*. ABCA12 failed to co-localize with TGase1, a cell membrane-bounding protein, both *in vivo* and in the cultured keratinocytes, and ABCA12 was thought to be distributed only very sparsely, on the cell membrane.

Furthermore, postembedding immunoelectron microscopy and cryoultramicrotomy revealed that ABCA12 was located on LG in the uppermost granular layer cells. Glucosylceramide was seen on the lamellar structures inside

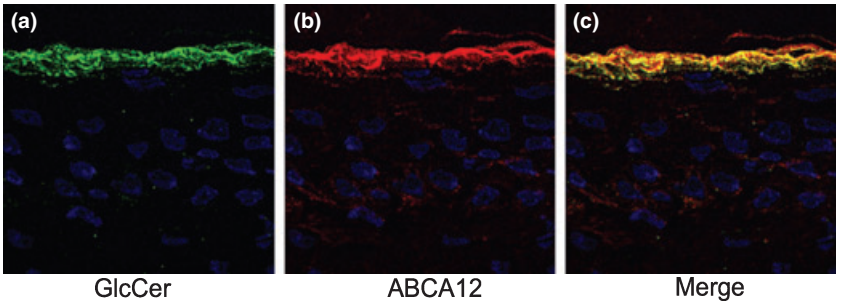

Figure 4. Immunofluorescence labelling using ultrathin cryosections for ABCA12 and glucosylceramide. Immunofluorescence labelling on ultrathin cryosections clearly demonstrated that glucosylceramide (a) and ABCA12 (b) overlapped in the cytoplasm of the granular layer cells (c). ABCA12, red (TRITC); GlcCer, green (fluorescein isothiocyanate); nuclear stain, blue (TO-PRO-3). Magnification ×100.

the LG and was subsequently secreted to the intercellular space. Taking all these findings into consideration, in the uppermost granular layer cells, ABCA12 was thought to be distributed to the Golgi apparatus limiting membrane and subsequently to LG containing glucosylceramide at the keratinocyte periphery. These results supported our hypothesis that *ABCA12* is working in lipid transport from the Golgi apparatus to LG in the uppermost granular layer cells (Fig. 6).

LG are known as lipid-transporting granules and LG contents are secreted to the intercellular space forming intercellular lipid layers between the stratum corneum corneocytes, which is essential for the skin barrier function. Recently, it was shown that LG are not true granules, but are tubular structures distended from the *trans*-Golgi network (34,41,42). LG may have their own unique trafficking and secretory systems. Considering our results that ABCA12 was localized from the Golgi apparatus to LG at the cell periphery, this supports the hypothesis that ABCA12 is likely to be a membrane lipid transporter that functions in the lipid transport from the *trans*-Golgi network to LG at the keratinocyte periphery. In this context, the LG abnormality in HI resulting from loss of ABCA12 function probably causes a blockade of the normal lipid transport and secretion and leads to congestion of the lipid flow within the keratinocyte.

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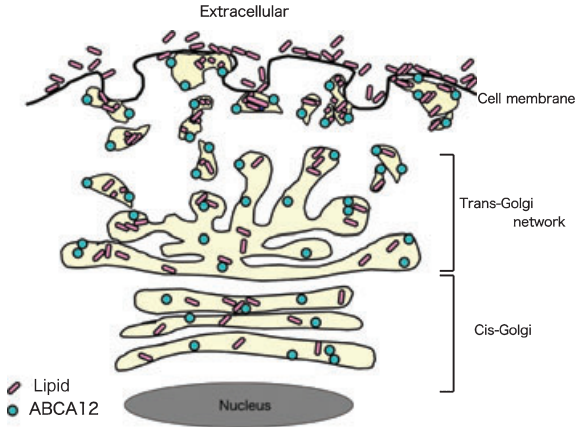

Figure 6. Hypothetical scheme of ABCA12 distribution at the apical border of differentiated epidermal keratinocytes. The lipid transporter ABCA12 was localized to the membrane from the Golgi apparatus to the lamellar granules (LG). ABCA12 is thought to be associated with lipid transport from the Golgi apparatus to cell periphery where these lipids are secreted from LG in the uppermost granular layer cells.

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