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Ishida-Yamamoto A, Kato H, Kiyama H, Armstrong DKB, Munro CS, Eady RAJ, Nakamura S, Kinouchi M, Takahashi H, Iizuka H

Mutant Loricrin is Not Crosslinked into the Cornified Cell Envelope but is Translocated into the Nuclei in Loricrin Keratoderma.

Akemi Ishida-Yamamoto,* Hidemasa Kato, Hiroshi Kiyama, D. Keith B. Armstrongà, Colin S. Munro, ¤ Robin A.J. Eady,¦ Satoshi Nakamura,* Motoshi Kinouchi,* Hidetoshi Takahashi,* and Hajime Iizuka*
*Department of Dermatology, and
Department of Anatomy, Asahikawa Medical College, Asahikawa, Japan
àDivision of Molecular Medicine, Queen's University Belfast, Belfast, Northern Ireland, U.K.
¤Department of Dermatology, Southern General Hospital, Glasgow, U.K.
¦Department of Cell and Molecular Pathology, St. John's Institute of Dermatology, GKT School of Medicine, St. Thomas's Hospital, London, U.K.

Short title: Nuclear localization of mutant loricrin
List of abbreviations: CE, cornified cell envelope; IVS, the ichthyotic variant of Vohwinkel syndrome; PSEK-PPK, progressive symmetric erythrokeratoderma with palmoplantar keratoderma; LK, loricrin keratoderma; PBS, phosphate buffered saline; TBS, Tris-HCl buffered saline; FCs, fibrillar centers; DFC, dense fibrillar component; GC, granular component;

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@mail.asahikawa-med.ac.jp

ABSTRACT
Loricrin is a major constituent of the epidermal cornified cell envelope (CE). We have recently identified heterozygous loricrin gene mutations in two dominantly inherited skin diseases, the ichthyotic variant of Vohwinkel syndrome (IVS) and progressive symmetric erythrokeratoderma with palmoplantar keratoderma (PSEK-PPK), collectively termed loricrin keratoderma (LK). In order to see whether the mutant loricrin molecules predicted by DNA sequencing are expressed in vivo and to define their pathological effects, we raised antibodies against synthetic peptides corresponding to the mutated sequences of loricrin. Immunoblotting of horny cell extracts from LK patients showed specific bands for mutant loricrin. Immunohistochemistry of LK skin biopsies showed positive immunoreactivity to the mutant loricrin antibodies in the nuclei of differentiated epidermal keratinocytes. The immunostaining was localized to the nucleoli of the middle epidermal layer. As keratinocyte differentiation progressed the immunoreactivity moved gradually into the nucleoplasm leaving nucleoli mostly non-immunoreactive. No substantial staining was observed along the CE. The present study confirmed that mutant loricrin was expressed in the LK skin. Mutant loricrin, as a dominant negative disrupter, is not likely to affect CE crosslinking directly, but seems to interfere with nuclear/nucleolar functions of differentiating keratinocytes. In addition, detection of the mutant loricrin in scraped horny layer could provide a simple non-invasive screening test for LK.

Key words: nuclear localization signals, nucleolus, palmoplantar keratoderma, progressive symmetric erythrokeratoderma, Vohwinkel syndrome

The cornified cell envelope (CE) is a covalently crosslinked layer of protein that is formed by epidermal keratinocytes in a late stage of differentiation (Ishida-Yamamoto and Iizuka, 1998). Loricrin is a small basic protein synthesized in the upper granular layer and becomes a major constituent of the CE (Mehrel et al., 1990; Hohl et al., 1991; Yoneda et al., 1992; Ishida-Yamamoto et al., 1998a). Heterozygous mutations in the loricrin gene have been detected in two forms of dominantly inherited skin diseases, the ichthyotic variant of Vohwinkel syndrome (IVS) and progressive symmetric erythrokeratoderma with palmoplantar keratoderma (PSEK-PPK) (Maestrini et al., 1996; Ishida-Yamamoto et al., 1997; Korge et al., 1997; Armstrong et al., 1998; Takahashi et al., 1999). These disorders have been collectively termed `loricrin keratoderma' (LK) (Ishida-Yamamoto and Iizuka, 1998; Ishida-Yamamoto et al., 1998a). The mutations found in LK are very similar in that they are insertions of a single nucleotide resulting in a frameshift and a delayed termination codon. The patients also share clinical features: mutilating palmoplantar hyperkeratosis with a "honeycomb" appearance and formation of digital constriction bands (pseudoainhum). Other clinical manifestations entail mild ichthyosis in IVS and generalized erythematous keratotic plaques in PSEK-PPK.

Although the gene defects underlying LK have been identified, the mechanism

by which the mutant loricrin causes keratoderma remains unknown. Since the cornified cells of the patients showed some characteristics of transitional cells (Ishida-Yamamoto et al., 1998b), we speculated that the differentiation of keratinocytes from the granular to the cornified cells is specifically disrupted at the transitional cell level. In the LK skin, loricrin was distributed aberrantly when it was stained with antibodies against the N-terminus of loricrin (Maestrini et al., 1996; Ishida-Yamamoto et al., 1997; Korge et al., 1997). Nuclear staining was stronger with the N-terminal loricrin antibody compared with the C-terminal antibody, suggesting that the mutant loricrin, which cannot be recognized by the C-terminal antibody, was preferentially localized within the nuclei in LK (Korge et al., 1997). In order to determine where the mutant loricrin is expressed in LK skin, we raised antibodies against the predicted C-terminal sequences that are common to all known loricrin mutants which could be analysed by immunohistochemistry. We found that the mutant loricrin was indeed expressed in LK skin and that its localization was exclusively in the nucleus rather than the CE. This suggests that mutant loricrin deranges keratinocyte terminal differentiation not by direct disruption of CE assembly, but by affecting nuclear function.

MATERIALS AND METHODS

Samples Normal human skin from the neck and the thigh was obtained at the time of plastic surgery. Biopsy from lesional skin of two patients with PSEK-PPK and a patient with IVS, and lesional and non-lesional skin of another patient with IVS were taken after obtaining informed consent. Heterozygous insertion mutations of the loricrin gene have been identified in these patients, a C insertion at codons 224-225 in PSEK-PPK and a T insertion at codon 209 and a G insertion at codons 230-231 in IVS (Ishida-Yamamoto, et al., 1997; Korge et al., 1997; Armstrong et al., 1998). Horny layers of four LK patients (two with PSEK-PPK and two with IVS) were also collected by scraping the sole or arm with a razor blade.

Production of mutant loricrin antibody C-terminal 18 amino acids of mutant loricrin plus cystein at the N-terminus was synthesized (CS Bio Co., San Carlos, CA). This peptide was covalently coupled to keyhole limpet hemocyanin (Pierce Chemical Co., Rockford, Ill) through the sulfhydryl group of the N-terminal cystein with the bifunctional crosslinker m-maleimidobenzoyl-N-hydroxysuccinimide ester (Boehringer-Mannheim, Tokyo, Japan) and used to immunize rabbits. Sera with high ELISA titer to the mutant loricrin peptide were obtained from two rabbits (mLo1, mLo2).

Immunoblot analysis Scraped stratum corneum cells from four LK patients and a normal human subject was fixed with 10% trichloroacetic acid in phosphate buffered saline pH7.4 (PBS) for 30 min on ice and cenrifuged at 1,000 rpm for 5 min at 4¡C. This pellet was sonicated and extracted in 9 mol/l urea, 2% TritonX-100, 1% dithiothreitol/Tris buffer. 10 mg of total protein was loaded on a lane of 1-mm-thick 12.5% SDS-polyacrylamide slab gels, and subjected to electrophoresis as described by Laemmli (Laemmli, 1970). After electrophoresis, gel slabs containing separated proteins were electrophoretically transferred to polyvinylidene difluoride membrane (Immobilon-P, Nihon Millipore, Tokyo, Japan) in transfer buffer containing 25 mM Tris and 192 mM glycine. Non-specific binding sites were blocked by immersing the membranes in 5% skim milk in Tris-HCl buffered saline pH7.6 (TBS) for 1 hour at room temperature. After rinsing in TBS, the membrane was incubated with mLo antisera (mLo1 and mLo2, both in 1:4000 dilution in TBS) or with antibody against C-terminus of normal loricrin (AF62, Berdeley Antibody Company, Richmond, California, 1:4000 dilution inTBS) overnight at 4¡C. After rinsing in TBS, the membrane was incubated with horseradish peroxidase-linked F(ab')2 fragment against rabbit Ig from donkey (Amersham, Buckinghamshire, UK, 1:5000 dilution in TBS). ECL+Plus western blotting detection system (Amersham) was used for immunodetection. For a competition analysis, the diluted primary antibodies were reacted with the synthetic mutant loricrin peptide (5 mM) for 1 hour at 37¡C. Molecular weight markers were: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000) and carbonic anhydrase (30,000). The experiments were performed in duplicate, each time essentially with the same results.

Immunofluorescence Immunofluorescence studies on semi-thin sections of Lowicryl-embedded skin (see below) and cryostat sections of the scraped skin were incubated with mLo antisera (mLo1 and mLo2, both in 1:100 dilution in PBS) or with antibody against C-terminus of normal loricrin (AF62, 1:500 dilution in PBS) for 30 min at 37¡C. This was followed by incubation with FITC-conjugated swine anti-rabbit

IgG antibodies (DAKO, Glostrup, Denmark) after dilution (1:20) in PBS for 30 min at 37¡C. For peptide competition, the diluted mLo antisera were preincubated with 5 mM peptide for 1 hour at 37¡C and then used for immunofluorescence. Nuclei were stained with propidium iodide.

Electron microscopy and immunoelectron microscopy Transmission electron microscopy was performed as described elsewhere (Ishida-Yamamoto et al., 1997; Korge et al., 1997). For immunoelectron microscopy, skin tissue samples were cryofixed, cryosubstituted and embedded in Lowicryl K11M resin (Chemische Werke Lowi, Waldkraiburg, Germany) according to the methods previously described (Shimizu et al., 1989; Ishida-Yamamoto et al., 1996). Ultrathin sections were cut, collected on formvar-coated nickel grids, and immunostained as described previously (Ishida-Yamamoto et al., 1996). As the primary antibodies mLo antisera (mLo1 and mLo2 both in 1:200 dilution), anti-loricrin antibody (AF62, 1:500 dilution) and anti-involucrin mouse monoclonal antibodies (SY5, kindly provided by Dr. FM Watt, 1:100 dilution) were used. As labels, 10 nm gold-conjugated goat anti-rabbit IgG and 5 nm gold-conjugated goat anti-mouse IgG (Amersham, 1:10 dilution) were used. To retrieve masked epitopes, ultrathin sections were incubated prior to immunostaining with 0.4 mg/ml proteinase K in 50 mM Tris¥HCl (pH 7.4) at room temperature for 30 sec to 5 min. For contrast, the sections were stained with uranyl acetate. For all immunohistochemistry, negative controls included incubation in the presence of a secondary antibody alone, and incubation with unrelated primary antibodies.

RESULTS

Mutant loricrin was detected in the loricrin keratoderma skin Two antibodies against the mutant loricrin peptide, mLo1 and mLo2, gave essentially the same results. Immunoblots of horny cell extracts from two PSEK-PPK patients showed a band of 39 kDa, but no band was detected in control samples (Fig 1). This reaction was greatly decreased when the mLo antibodies were pre-incubated with mutant loricrin peptides. Higher molecular weight bands of about 80 and 120 kDa were also detected in the patient samples. We did not detect apparent difference in the amount of expressed mutant loricrin proteins between the horny layer samples from two PSEK-PPK patients and from two IVS patients (not shown). No band was detected for the antibody against normal C-terminus of loricrin (not shown).

Lesional skin from the four LK patients showed identical immunostaining patterns (Fig. 2). Positive reaction was detected almost exclusively within the nuclei of differentiated epidermal keratinocytes. In a higher magnification immunoreactivity was found to be associated with distinct globular structures in the middle epidermal layers, while large areas of nucleoplasm became immunoreactive in the superficial cell layers. This staining was abolished by preincubating the mLo antibodies with the mutant loricrin peptide. The antibodies also stained parakeratotic nuclei of the scraped horny layer of LK skin. Positive reaction was also obtained in the nuclei of upper granular cells and cornified cells from non-lesional skin of a LK patient. Normal control skin did not show immunoreactivity. The antibody against C-terminus of wild-type loricrin showed positive reactions in the cytoplasm as well as in the nucleus in the granular cells of both normal and LK epidermis.

Mutant loricrin was associated with nucleolus To correlate immunohistochemical findings with morphological events in the nuclei and to define localization of mutant loricrin at an ultrastructural level, transmission electron microscopy and post-embedding immunoelectron microscopy were performed. In both normal and LK epidermis, nuclei in the basal, spinous and lower granular cells were with little heterochromatin and active large reticulate nucleoli that contain several fibrillar centers (FCs) surrounded by dense fibrillar component (DFC) and granular component (GC) (Fig. 3). Nuclei in the upper granular cells had increased heterochromatin and a small non-active nucleolus with a few FCs. Electron dense granular deposit in the nucleoplasm and within the nucleoli of the middle to upper granular cells was unique to LK skin. In the lesional skin, mLo staining was detected within the nuclei from the lower granular cells through the cornified cells (Fig. 4). It was specifically on GC and DFC of active nucleolus in the lower granular cells. In the middle granular cells, immunopositive deposit appeared around the nucleolus and also in the nucleoplasm. In the superficial granular cells, most of the labels were in the nucleoplasm, while nucleolus became mostly non-immunoreactive. In the non-lesional skin, the uppermost granular cells contained immunoreactive deposit in the nucleoplasm. Nucleolus were usually inactive ones with some positive immunoreaction.

Anti-normal loricrin antibodies stained diffusely the granular cell cytoplasm and nuclei. It was not particularly associated with nucleoli in the normal skin nor in the LK skin.

Normal loricrin epitopes on the cornified cell envelopes were masked The CE in the cornified cells of the LK patients was strongly positive to involucrin antibodies, but showed very little labeling for normal or mutant loricrin (Fig. 5). Since immature CE or cell membrane of the uppermost LK granular cells was relatively well labeled with antibodies against normal loricrin (Korge et al., 1997),we tested whether the decreased loricrin labeling in the CE of cornified layer was due to epitope masking. To retrieve possible masked epitopes, we performed immunoelectron microscopy on protease-treated sections (Ishida-Yamamoto et al., 1999). Loricrin labeling on the CE of LK cornified cells was greatly enhanced by protease K-digestion (for 30 sec to 5 min). CE remained non-immunoreactive to mLo antibodies after this treatment (not shown).

DISCUSSION

So far three types of loricrin mutations have been detected in genomic DNA from five LK families (Maestrini et al., 1996; Ishida-Yamamoto et al., 1997; Korge et al., 1997; Armstrong et al., 1998; Takahashi et al., 1999). All three mutations are heterozygous insertion mutations of a single nucleotide that result in a frameshift and a common downstream delayed termination codon.

In the present study, we raised antibodies against mutant loricrin. Immunobloting showed that the antibodies were specific and confirmed that mutant loricrin molecules were actually present in LK horny cells. The detected bands were of 39 kDa, larger than the molecular weight of 32 kDa calculated form mutant loricrin sequences (a C insertion at codons 224-225). Immunoblotting of normal loricrin showed also higher bands (32 kDa) than the calculated molecular weight (26 kDa) ((Hohl et al., 1993) and our own observation, not shown). These phenomena are probably due to the sequences rich in hydrophobic amino acids in both normal and mutant loricrin. The higher bands of 80 and 120 kDa are likely to be polymers of mutant loricrin.

Immunohistochemisty further demonstrated that mutant loricrin was expressed in the LK epidermis. The staining patterns were identical between the two IVS patients and two PSEK-PPK patients. The staining was localized in the differentiated keratinocytes in a predicted tissue- and differentiation-dependent manner and detected up to the cornified layer. Our results suggest that detection of the mutant loricrin in scraped horny layer by immunoblotting or immunohistochemistry might offer simple non-invasive screening tests for LK. We have recently tested a different family with PPK with these antibodies and obtained positive results. Subsequent DNA sequencing detected an insertion mutation of single nucleotide in the loricrin gene (unpublished).

As for the pathomechanisms of dominantly inherited disorders, some are explained as dominant negative effects of mutant molecules and others are attributed to haploinsufficiency. Although loricrin consists about 70% of CE in normal skin (Steven and Steinert, 1994; Steinert, 1995), the phenotype of loricrin null mice was just temporary and lasted only 4 - 5 days after birth1. This suggests that LK is not caused by haploinsufficiency of loricrin, but by some dominant negative effects of the mutant loricrin. The nature of this dominant negative effects remains unknown. We previously raised a possibility that the mutant loricrin disrupts assembly of CE (Maestrini et al., 1996; Ishida-Yamamoto et al., 1997; Armstrong et al., 1998). Because of the frameshift mutations, mutant molecules lack the C-terminal glutamine-lysine rich domain essential for crosslinking. CE of the LK cornified cells was immunoreactive to involucrin antibodies, but showed very little immunoreactivities to loricrin antibodies (Maestrini et al., 1996; Ishida-Yamamoto et al., 1997; Korge et al., 1997). This is contrary to the normal CE that was positive to loricrin, but negative to involucrin (Ishida-Yamamoto et al., 1996). However, wild-type loricrin was detected along the CE in the granular cells of LK and loricrin epitopes on the CE of cornified cells were retrieved by protease digestion of the sections. Therefore, it is more likely that normal loricrin is crosslinked into CE of LK, but its epitopes are masked by extensive further crosslinking with other molecules such as involucrin. Involucrin is one of the early constituents of CE (Eckert et al., 1993; Robinson et al., 1996; Steinert and Marekov, 1997) with their epitopes normally masked when the CE is matured by later crosslinking of loricrin and other molecules (Ishida-Yamamoto et al., 1996). By contrast, involucrin crosslinking seems to continue after loricrin is crosslinked and mask loricrin epitopes in LK.

Because mutant loricrin was expressed within the nuclei and not associated with cell membranes or CE, it is unlikely that the mutant loricrin is crosslinked into CE directly interfering with its formation. Instead, it is plausible to speculate that mutant loricrin deranges keratinocyte terminal differentiation by some other mechanisms. Nuclear localization of mutant loricrin suggests that it exerts dominant negative effects through interactions with nuclear proteins and/or nucleic acids. Interestingly arginine-rich sequences of mutant loricrin shows homology to a highly basic nuclear protein, protamines (43% identities at the amino acid level with sperm protamine SP1) (Willmitzer and Wagner, 1980; Wouters-Tyrou et al., 1998). It is known that protamines interact with DNA on their arginine clusters. Alternatively initial association with nucleoli might indicate a pathological role of the mutant loricrin upon nucleolar functions.

In the LK epidermis, nucleoli from the basal to the lower granular layer were not apparently different from those in normal skin, but those in the upper layer were distinct. There was deposition of mutant loricrin within and around the nucleoli. Localization of mutant loricrin in the DFC and GC of the cells with a high level of ribosomal biosynthesis suggests interaction between mutant loricrin and rRNA or rRNA binding proteins. Recent evidence also suggests that nucleolus is a preferred intracellular target for apoptotic events (Stegh et al., 1998). Terminal differentiation of keratinocytes is a typical example of programmed cell death (Ishida-Yamamoto et al., 1998b). Mutant loricrin might derange keratinocyte differentiation/cell death pathway by affecting nucleolus as a target of apoptotic cellular changes. This might explain delay of cell death process in LK epidermis in the stage where nuclear DNA is fragmented (Ishida-Yamamoto et al., 1998b). Alternatively mutant loricrin might disturb other functions of nucleolus that include non-ribosomal RNA processing and growth factor signal transduction (Pederson, 1998a; Pederson, 1998b).

The reason for limited distribution of skin lesions in LK patients despite the generalized expression of the mutant loricrin is obscure at this moment. In the non-lesional skin mutant loricrin-expressing cells were just one cell layer-thick and were to cornify in the next cell layer. It might be that if mutant loricrin is expressed in a very late stage of terminal differentiation where nucleolus is no more active, the mutant loricrin does not hamper the completion of differentiation. In the palmoplantar skin loricrin-expressing cells are several layer-thick. When the cells start to express mutant loricrin, they still have a long way to go with late keratinization processes that are more likely to be affected. It remains to be explained why the extra-palmoplantar lesions vary in PSEK-PPK and IVS as well.

Small proteins with molecular mass less than 40 - 60 kDa can cross the nuclear pore complex by diffusion (Nigg, 1997). Loricrin is a small molecule (26 kDa in human and 38 kDa in mouse) and localized both in the cytoplasm and nucleoplasm (Steven et al., 1990; Ishida-Yamamoto et al., 1996; Ishida-Yamamoto et al., 1998a). The mechanisms for nuclear translocation and specific association with nucleolus of mutant loricrin remain unknown at present. The sequences of mutant loricrin rich in arginine might function as consensus bipartite nuclear localization signals (Korge et al., 1997). Nucleolar protein localization system is not well understood and it has been suggested that a common motif to some nucleolar targeting sequences is short highly basic segments rich in arginine or lysine. A peptapeptide (RRQRR), which has been implicated as part of a nucleolar targeting element in signal recognition particle (Pederson, 1998b), is present in the mutant loricrin and this might be functional. These possibilities have to be tested in the future studies.

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REFERENCES

Armstrong DKB, McKenna KE, Hughes AE: A novel insertional mutation in loricrin in Vohwinkel's keratoderma. J Invest Dermatol. 111:702-704, 1998.

Eckert RL, Yaffe MB, Crish JF, Murthy S, Rorke EA,Welter JF: Involucrin - structure and role in envelope assembly. J Invest Dermatol 100:613-617, 1993.

Hohl D, Mehrel T, Lichti U, Turner ML, Roop DR, Steinert PM: Characterization of human loricrin. Structure and function of a new class of epidermal cell envelope proteins. J Biol Chem 266:6626-6636, 1991.

Hohl D, Olano BR, de Viragh PA, Huber M, Detrisac CJ, Schnyder UW, Roop DR: Expression patterns of loricrin in various species and tissues. Differentiation 54:25-34, 1993.

Ishida-Yamamoto A, Eady RAJ, Watt FM, Roop DR, Hohl D, Iizuka H: Immunoelectron microscopic analysis of cornified cell envelope formation in normal and psoriatic epidermis. J Histochem Cytochem 44:167-175, 1996.

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

Ishida-Yamamoto A, McGrath JA, Lam H, Iizuka H, Friedman RA, Christiano AM: The molecular pathology of progressive symmetric erythrokeratoderma: a frameshift mutation in the loricrin gene and perturbations in the cornified cell envelope. Am J Hum Genet 61:581-589, 1997.

Ishida-Yamamoto A, Takahashi H, Iizuka H: Loricrin and human skin disease: molecular basis of loricrin kratodermas. Histol Histopath 13:819-826, 1998a.

Ishida-Yamamoto A, Takahashi H, Presland RB, Dale BA, Iizuka H: Translocalion of profilaggrin N-terminal domain into keratinocyte nuclei with fragmented DNA in normal human skin and loricrin keratoderma. Laboratory Investigation 78:1245-1253, 1998b.

Korge BP, Ishida-Yamamoto A, Punter C, et al.: Loricrin mutation in Vohwinkel's keratoderma is unique to the variant with ichthyosis. J Invest Dermatol 109:604-610, 1997.

Laemmli UK: Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature 227:680-685, 1970.

Maestrini E, Monaco AP, McGrath JA, et al.: A molecular defect in loricrin, the major component of the cornified cell envelope, underlies Vohwinkel's syndrome. Nature Genet 13:70-77, 1996.

Matsuki M, Yamashita F, Ishida-Yamamoto A, et al.: Defective stratum corneum and early neonatal death in mice lacking the gene for transglutaminase 1 (keratinocyte transglutaminase). Proc Natl Acad Sci 95:1044-1049, 1998.

Mehrel T, Hohl D, Rothnagel JA, et al.: Identification of a major keratinocyte cell envelope protein, loricrin. Cell 61:1103-1112, 1990.

Nigg EA: Nucleocytoplasmic transport: signals, mechanisms and regulation. Nature 386:779-787, 1997.

Pederson T: Growth factors in the nucleolus? J Cell Biol 143:279-281, 1998a.

Pederson T: The plurifunctional nucleolus. Nucleic Acid Res 26:3871-3876, 1998b.

Robinson NA, LaCelle PT, Eckert RL: Involucrin is a covalently crosslinked constituent of highly purified epidermal corneocytes: Evidence for a common pattern of involucrin crosslinking in vivo and in vitro. J Invest Dermatol 107:101-107, 1996.

Shimizu H, McDonald JN, Kennedy AR, Eady RAJ: Demonstration of intra- and extracellular localization of bullous pemphigoid antigen using cryofixation and freeze substitution for postembedding immunoelectron microscopy. Arch Dermatol Res 281:443-448, 1989.

Stegh AH, Schickling O, Ehret A, et al.: DEDD, a novel death effector domain-containing protein, targeted to the nucleolus. EMBO j 17:5974-5986, 1998.

Steinert PM: A model for the hierarchical structure of the human epidermal cornified cell envelope. Cell Death and Differentiation 2:33-40, 1995.

Steinert PM, Marekov LN: Direct evidence that involucrin is a major early isopeptide

cross-linked component of the keratinocyte cornified cell envelope. J Biol Chem 272:2021-2030, 1997.

Steven AC, Bisher ME, Roop DR, Steinert PM: Biosynthetic pathways of filaggrin and loricrin - two major proteins expressed by terminally differentiated epidermal keratinocytes. J Struct Biol 104:150-162, 1990.

Steven AC, Steinert PM: Protein composition of cornified cell envelopes of epidermal keratinocytes. J Cell Sci 107:693-700, 1994.

Takahashi H, Ishida-Yamamoto A, Kishi A, Ohara K, Iizuka H: Loricrin gene mutation in a Japanese patient of Vohwinkel's syndrome. J Dermatol Sci 19:44-47, 1999.

Willmitzer L,Wagner KG: The binding of protamines to DNA; role of protamine phosphorylation. Biophys Struct Mech 6:95-110, 1980.

Wouters-Tyrou D, Martinage A, Chevaillier P, Sautiere P: Nuclear basic proteins in spermiogenesis. Biochimie 80:117-128, 1998.

Yoneda K, Hohl D, McBride OW, Wang M, Cehrs KU, Idler WW, Steinert PM: The human loricrin gene. J Biol Chem 267:18060-18066, 1992.

Figure Legend

Figure 1. Immunoblot detects mutant loricrin. Positive bands are detected in LK horny cells (lanes 2 and 3), but not in normal control (lane 1). Extracts from scraped horny cells were fractionated on 12.5% SDS-polyacrylamide gels and immunoblotted with the mLo2 antibody pre-incubated with 5 mM mutant loricrin peptide in TBS (lane 3) or with TBS (lanes 1 and 2) for 1 hour at 37¡C. The sizes of molecular weight standards are shown on the left. The arrows on the right denote the approximately 39-kDa mutant loricrin protein and possible its polymers.

Figure 2. Mutant loricrin is expressed within the nuclei of LK keratinocytes. Mutant (a to f) and wild-type loricrin (g) staining. Lesional skin (a to c, g), scraped sole skin horny layer (d) and non-lesional skin (e) from LK patients and normal control skin (f). (a) Green fluorescence for mutant loricrin is detected within the differentiated keratinocyte nuclei. (b) Mutant loricrin is associated with globular intranuclear structures in the middle epidermal cells (arrow heads). (c) Preincubation of the antibody with mutant loricrin peptide abolishes immunoreaction. (d) Punctate staining in the scraped horny layer of LK sole skin. (e) The most-superficial granular cells in the non-lesional skin also express mutant loricrin. (f) No reaction is detected in normal skin. (g) Normal loricrin is expressed in the cytoplasm as well as in the nucleus. a, c, d, f X370. b, e, g X740. Bars = 10 mm.

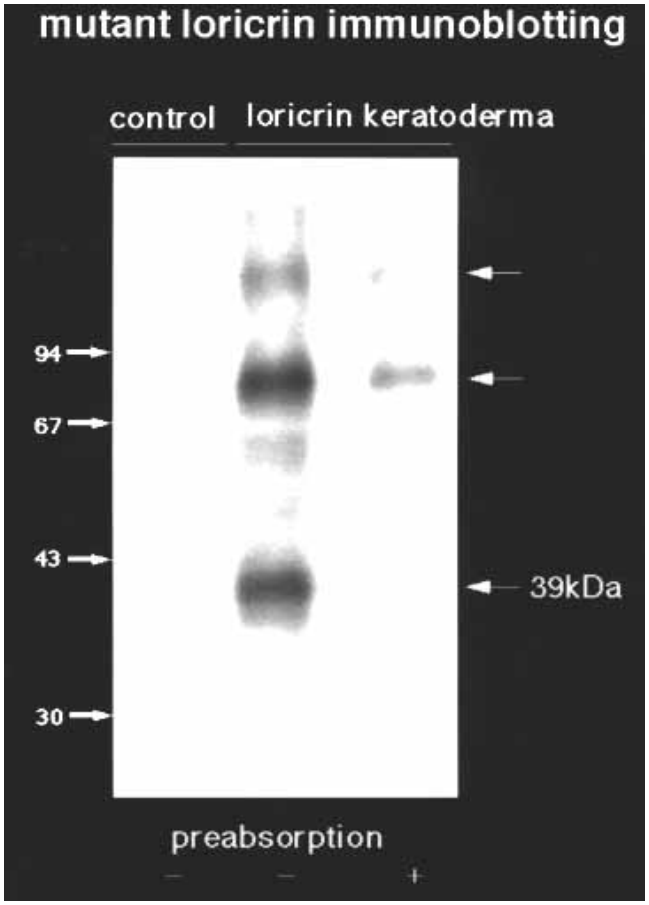
Figure 3. Electron dense nuclear granules appear around an inactive nucleolus in the differentiated LK keratinocytes. Transmission electron microscopy of normal (a, c) and LK epidermis (b, d). (a, b) Active large reticulate nucleoli in the lower granular cells contain several fibrillar centers (c) surrounded by dense fibrillar component (d) and granular component (g). (c, d) Nuclei in upper granular cells have a small non-active small nucleolus. Electron dense nuclear granules are unique to LK skin (d, arrowheads). Each nucleoli is shown in a lower magnification as an inset. ch, chromatin. X 19,600. insets X2,450. Bar = 0.5 mm. Bar = 5 mm in insets.

Figure 4. Mutant loricrin is associated with an active-nucleolus. Immunoelectron microscopy of mutant (a, b) and wild-type loricrin (c) in LK epidermis. Nucleoli (arrows) in lower granular cells (a, c) and an upper granular cell (b). (a) Mutant loricrin is associated with dense fibrillar component (d) and granular component (g) of an active nucleolus. Fibrillar centers (c) are not labeled. (b) Mutant loricrin is on the periphery of an inactive nucleolus and in the nucleoplasm (arrowheads). (c) Wild-type loricrin is distributed diffusely, not particularly associating with nucleoli. X 40,000. Bar = 0.1 mm.

Figure 5. Mutant loricrin is not crosslinked into CE, while wild-type loricrin is crosslinked but epitopes are masked. Immunoelectron microscopy for involucrin (5 nm gold) and mutant loricrin (10 nm gold) (a) and wild-type loricrin (b, c). Un-digested (a, b) and proteinase K-digested (5 min) LK skin (c). (a) Along CE, there are many involucrin labels (arrowheads), but few labels for mutant loricrin (arrow). (b) Wild-type loricrin labeling is seen along the cell membrane (arrowheads) of the most

superficial granular cell (G), but not along the CE of the horny layer (H). (c) Proteinase K digestion has retrieved masked loricrin epitopes on the CE. X 56,000 (a). X 40,000 (b, c). Bars = 0.1 mm.

1 de Viragh PA, L. S, Bundman D, Roop DR: Transient congenital erythroderma in loricrin deficient mice. J Invest Dermatol 107:448, 1996 (abstr.)


mutant loricrin immunoblotting
control
loricrin keratoderma
94
67
43
30
39kDa
preabsorption
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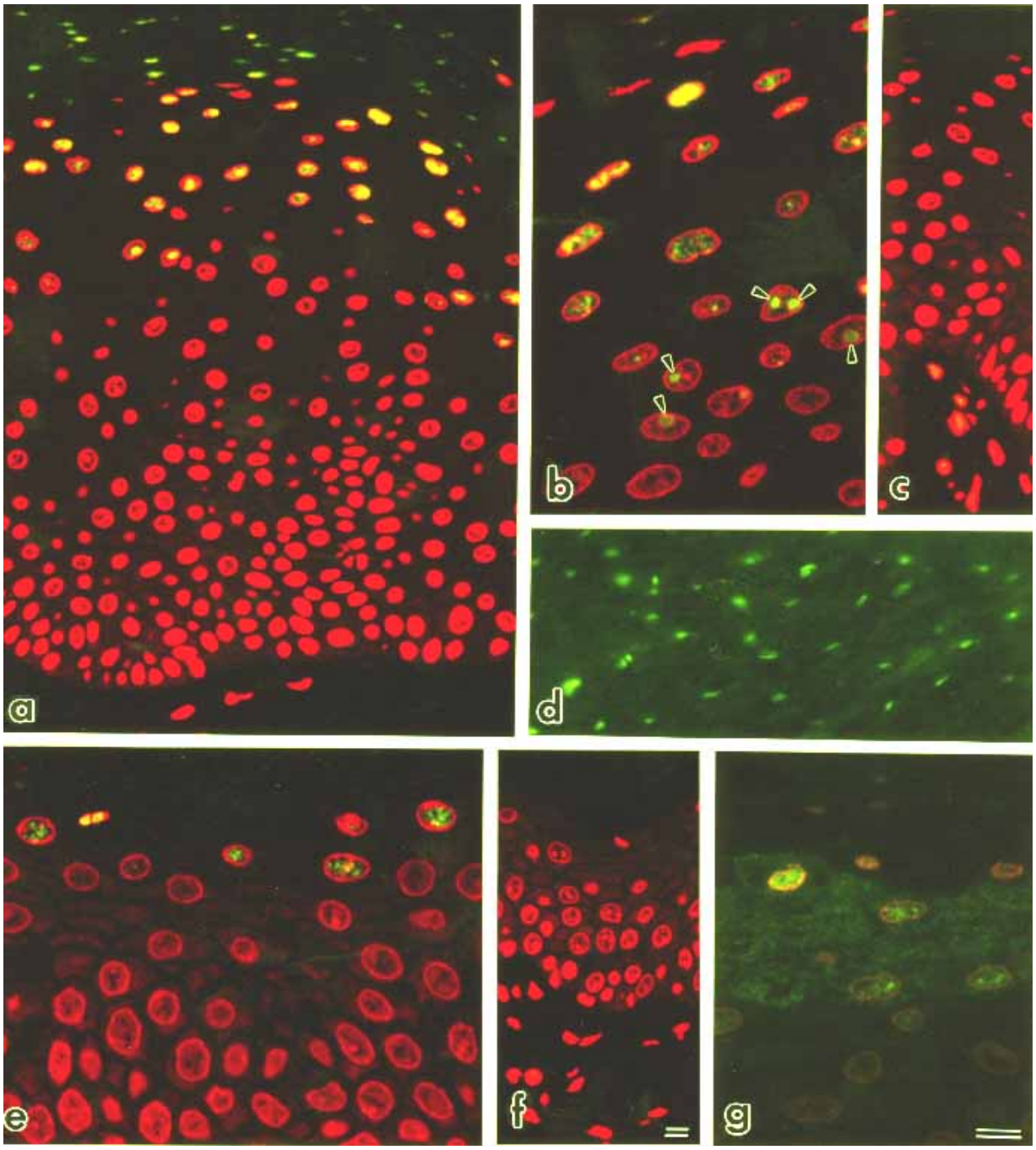
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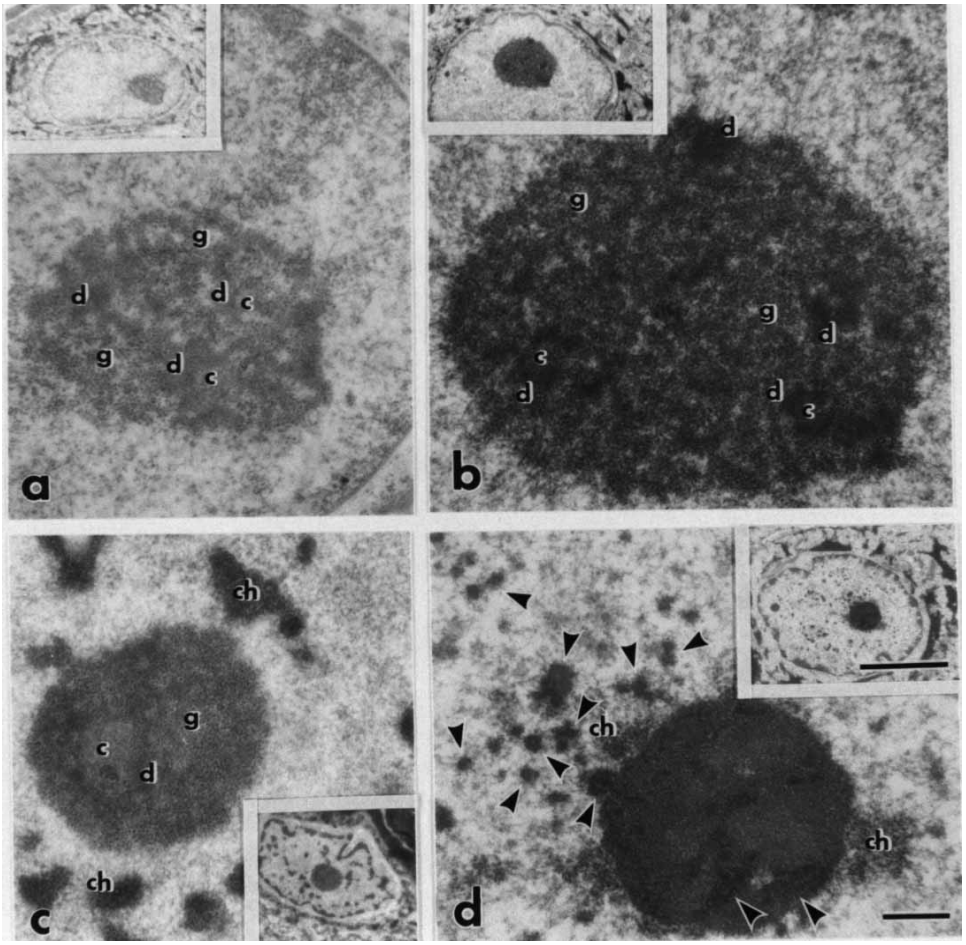
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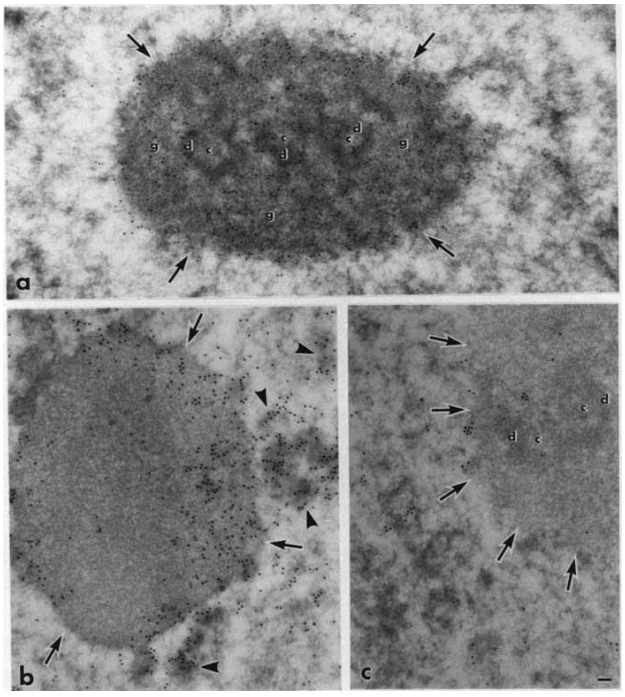
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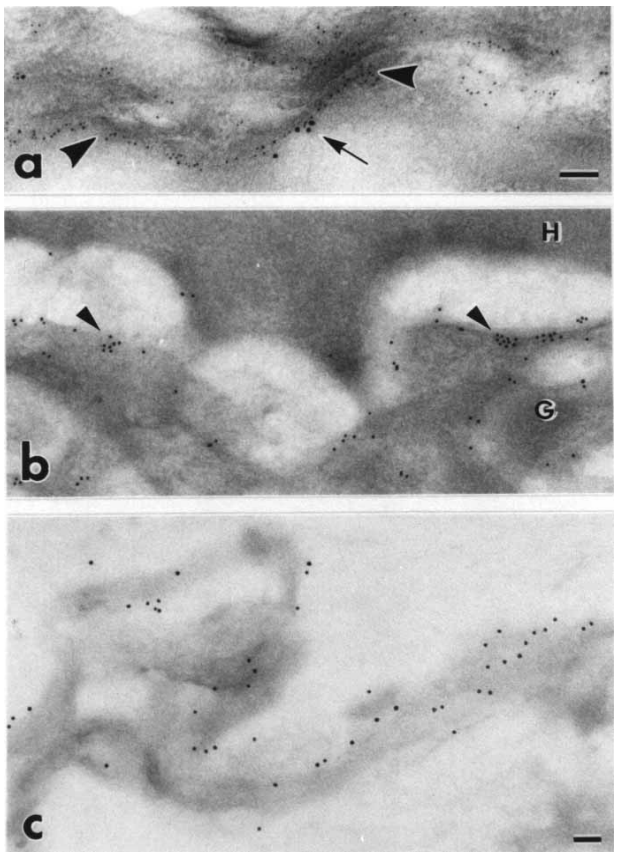
a
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