Histol Histopathol (1998) 13: 819-826

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Invited Review

Loricrin and human skin diseases: molecular basis of loricrin keratodermas

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Summary. The cornified cell envelope (CE) is a tough structure formed beneath the plasma membrane of terminally differentiated keratinocytes. Recent progress in understanding the molecular organization of the CE has disclosed the complex, yet orderly structure that functions as a protective barrier against the environment. We have recently demonstrated that two inherited skin diseases, Vohwinkel's syndrome (VS) and progressive symmetric erythrokeratoderma (PSEK) may result from mutations in the gene encoding loricrin, a major constituent of the CE. In adult human epidermis, loricrin is diffusely distributed within the superficial granular cells. In the cornified cells, loricrin is associated with CEs. In some patients with VS and PSEK skin, however, granular cells contain many intranuclear granules which are labeled with an amino-terminal loricrin antibody. CEs are thinner than normal and sparsely labeled with the loricrin antibody. Parakeratotic cornified cells contain loricrin-positive granules. Sequencing of the loricrin gene has disclosed heterozygous mutations; insertion of one nucleotide (730insG, 709insC) that shifts the reading frame in these patients. Consequently the carboxyl-terminus are replaced by highly charged missense sequences that override the endogeneous termination codon extending the protein with an additional 22 amino acids. Elucidation of the molecular biology of "loricrin keratodermas" adds to our understanding of the complexity and biological significance of the CE.

Key words: Cornified cell envelope, Inherited skin diseases, Keratinocytes, Progressive symmetric erythrokeratoderma, Vohwinkel's syndrome

Introduction

A hallmark of terminally differentiated or cornified epidermal keratinocytes is the formation of a cornified cell envelope (CE) (Hohl, 1990; Polakowska and Goldsmith, 1991; Reichert et al., 1993; Simon, 1994; Eckert et al., 1997; Ishida-Yamamoto and Iizuka, 1998). The CE is a continuous structure, 15- to 20-nm in thickness, formed beneath the plasma membrane (Brody, 1969). It constitutes the most insoluble structure of stratified squamous epithelia and provides the tissue with a protective barrier against the environment. The CE is composed of several molecules, including involucrin, cystatin A, elafin, loricrin and SPRRs. These molecules are cross-linked with Nε-(γ-glutamyl) lysine isopeptide bonds via the action of transglutaminases 1 and 3.

Although the composition of the constituent proteins of CE varies in different epithelia, loricrin (in latin, lorica stands for protective cuirasse of leather and metal) is most abundant in the epidermal CE (Steven and Steinert, 1994; Steinert, 1995). The physiological and pathological significance of loricrin has been highlighted by recent discoveries of mutations in the loricrin gene in two inherited skin disorders, Vohwinkel's syndrome (VS) and progressive symmetric erythrokeratoderma (PSEK) (Maestrini et al., 1996; Ishida-Yamamoto et al., 1997; Korge et al., 1997). This article reviews the current understanding of the loricrin molecule and its gene, and details the clinicopathological findings and molecular genetic basis of the inherited diseases of loricrin.

Loricrin is a unique structural protein

Loricrin is a glycine-, serine- and cysteine-rich protein (Fig. 1) (Mehrel et al., 1990; Hohl et al., 1991b; Yoneda et al., 1992a). It is highly insoluble perhaps because of the high glycine-content and intra-molecular disulfide bonds. The human loricrin protein is 26 kDa, consisting of 315 amino acid residues with a calculated pI of 12 (Hohl et al., 1991b). In contrast, mouse loricrin is 38 kDa, contains 480 amino acids and has an estimated pI of 9.5 (Mehrel et al., 1990).

Loricrin contains many tandem quasi-repeats of a unique, highly flexible structure called a "glycine loop". Its general structure is represented as X(Y)n where X refers to aromatic or aliphatic amino acids, Y is glycine,

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serine or cysteine, and n is 1 to 35. Neither the sequences of these repeating peptides nor the number of glycine loops are conserved between mouse and human loricrin. Several sequence variations in the glycine loop domain of human loricrin have also been detected, suggesting that a limited degree of variation in the flexible glycine loops does not alter the function of CE (Yoneda et al., 1992a,b).

The glycine-loop domains are interrupted several times with glutamine-rich domains. The amino- and carboxyl-terminal domains of loricrin are rich in glutamine and lysine, and are highly conserved among different species and even have some homology to those of involucrin and SPRR (Backendorf and Hohl, 1992; Gibbs et al., 1993).

The loricrin gene

Loricrin is encoded by a single copy gene that consists of two exons and one intron (Mehrel et al., 1990; Hohl et al., 1991b; Yoneda et al., 1992a). The untranslated first exon is very short and the second exon contains the entire coding region. This gene configuration is very similar to that of involucrin (Eckert and Green, 1986). Loricrin mRNA is 1.6 kb in mouse and 1.3 kb in human. The human loricrin gene has been mapped to the epidermal differentiation complex (EDC) that is located on chromosome 1q21 together with several further genes expressed in differentiating keratinocytes (Volz et al., 1993; Mischke et al., 1996). Among these are the genes for involucrin, SPRRs, profilaggrin, trichohyalin and S100 family proteins. Because of their similarities to involucrin and SPRRs at both protein (see above) and gene structure levels, it has been assumed that these genes have evolved from a single ancestral gene (Backendorf and Hohl, 1992; Gibbs et al., 1993). The mouse loricrin gene has been mapped to the central region of chromosome 3 (Rothnagel et al., 1994). It is within 1.5±1.1 centimorgans of the profilaggrin loci and in the vicinity of the flaky tail and soft coat loci (Rothnagel et al., 1994).

The human loricrin gene with flanking sequence shows calcium-responsiveness and has been implicated in directing tissue- and differentiation-specific expression in transgenic mice (Yoneda and Steinert, 1993). Analysis of the loricrin promoter in cultured cells and transgenic mice has shown that the elements directing keratinocyte-specific expression can be segregated from those required for differentiation-specific expression (DiSepio et al., 1995). A 6.5-kb mouse genomic loricrin fragment which contains 1.5 kb of 5'-flanking sequence, two exons separated by an intron, and 2.2 kb of 3'-flanking sequence is expressed only in tissues normally expressing loricrin. However, the transgene is expressed not only in the differentiated suprabasal layer, but also in the basal layer in the transgenic mice epidermis. A larger clone of 14 kb, that contains an additional 6 kb of 5' and 0.5 kb of 3' sequence, was found to be expressed normally (i.e. suprabasally) in the differentiated keratinocytes only (DiSepio et al., 1994).

Tissue and cellular distribution of loricrin

Loricrin is expressed in the granular layer of orthokeratotic squamous epithelia (Mehrel et al., 1990; Steven et al., 1990; Hohl et al., 1991b) (Fig. 2). It is expressed at the final stage during the process of epithelial differentiation and occurs later than involucrin and profilaggrin expression. The intensity of loricrin expression varies in different body sites: it is abundant in foreskin and perianal skin, but less so in leg skin (Hohl, 1993).

In newborn mouse epidermis, loricrin accumulates in non-membrane-bound small round granules (L-granules) in the granular layer before being incorporated

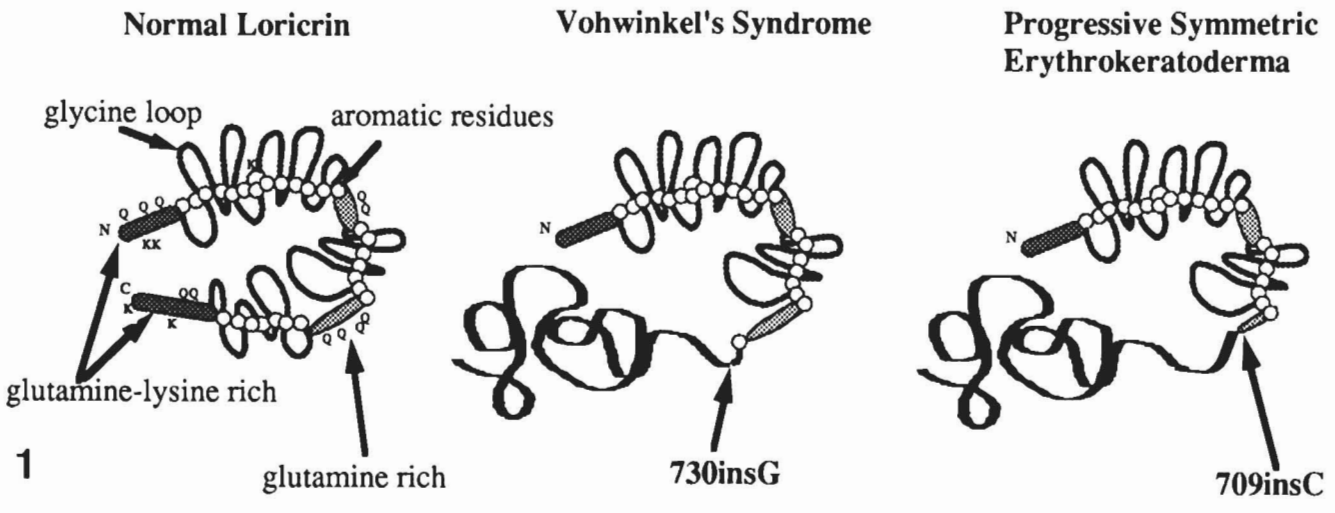

Fig. 1. Loricrin molecules in normal human skin and disease. Q and K denote glutamine and lysine residues where cross-linkage has been detected.

into the CE (Steven et al., 1990). In adult human epidermis, loricrin is diffusely distributed within the superficial granular cells with some accumulation at desmosomes (Ishida-Yamamoto et al., 1996). L-granules are scarcely found in adult human skin except for foreskin and acrosyringia (Ishida-Yamamoto et al., 1993, 1996). In acrosyringia, L-granules are seen as eosinophilic granules distinct from the basophilic filaggrin-containing keratohyalin granules (F-granules) (Ishida-Yamamoto et al., 1993).

In the cornified cells, loricrin is associated with CEs. Early CEs are involucrin-positive, but later its reactivity is lost, probably because its epitopes are masked with other CE precursor molecules including loricrin, that cross-link later than involucrin (see below) (Ishida-Yamamoto and Iizuka, 1995; Ishida-Yamamoto et al., 1996).

During the development of epidermis, loricrin mRNA/protein expression begins at about embryonic day 16 in mice (Yoneda and Steinert, 1993; Bickenbach et al., 1995) and by the mid second trimester in human (Holbrook et al., 1991; Akiyama et al., 1997). Loricrin was not detected in the periderm in mice (Bickenbach et al., 1995), but inconsistent detection patterns were observed in human fetal skin (Holbrook et al., 1991; Akiyama et al., 1997).

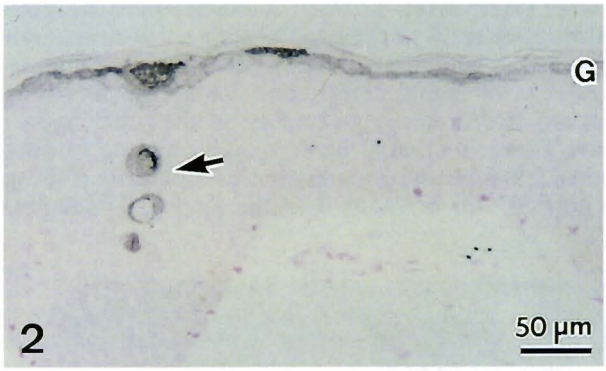

Fig. 2. Loricrin immunohistochemistry in normal human skin. Note positive staining in the epidermal granular layer (G) and acrosyringium (arrow). x 370

Regulation of loricrin expression and its alteration in skin diseases

In situ hybridization analysis has shown that loricrin mRNA transcripts are localized in the terminally differentiated keratinocytes of stratified squamous epithelia i.e. in the upper spinous layer and granular layer in the epidermis (Mehrel et al., 1990; Hohl et al., 1991b). The expression of loricrin protein starts slightly later in more differentiated cells (see above), suggesting that the loricrin expression is regulated at both transcriptional and posttranscriptional levels. When mouse loricrin was expressed throughout the entire epidermis in transgenic mice no phenotypic abnormalities were observed (DiSepio et al., 1994). Therefore, the expression of loricrin per se does not seem to initiate the terminal differentiation of keratinocytes. Similarly, overexpression of human loricrin in differentiated mice epidermis does not produce abnormal phenotypes (Yoneda and Steinert, 1993).

Transcription of the loricrin gene is up-regulated by calcium, protein kinase C and cell density, and repressed by retinoic acid *in vitro* (Hohl et al., 1991a; Magnaldo et al., 1992; Dlugosz and Yuspa, 1993; Brown et al., 1994). Although an AP-1 element in the 5'-proximal promoter is critical for transcription of the loricrin gene, synergistic effects of both 5'- and 3'-regulatory elements are also required (DiSepio et al., 1995). Loricrin can be induced in epithelia that do not normally express loricrin. For example, squamous metaplasia induced by estrogen and by vitamin A deficiency in rat vaginal epi-

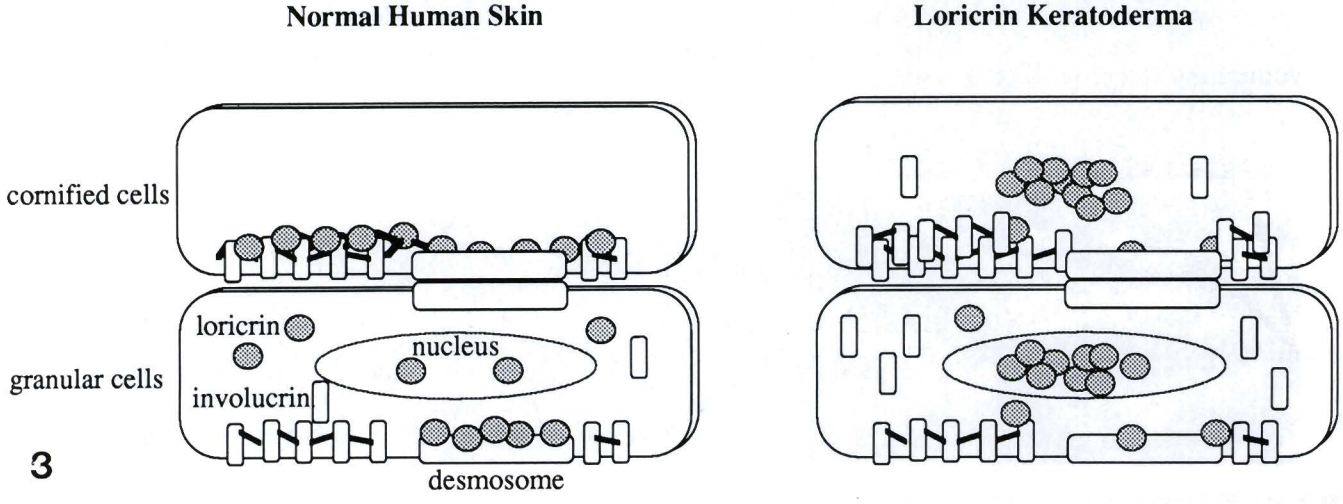

Fig. 3. Cornified cell envelope (CE) formation in normal human skin and in loricrin keratoderma. (Only involucrin and loricrin are depicted for simplicity.)

thelium and hamster tracheal epithelium, respectively, is accompanied by loricrin expression (Hohl et al., 1993).

Loricrin expression is decreased in situations of hypo- or agranulotic parakeratotic keratinization including psoriasis (Juhlin et al., 1992; Hohl, 1993; Takahashi et al., 1996), dermatitis, pityriasis lichenoides, porokeratosis and precancerous and malignant squamous lesions (Hohl, 1993). High levels of loricrin are found in hypergranulotic and orthokeratotic lesions in lichen planus, benign papillomas and pseudocarcinomatous hyperplasia (Hohl, 1993). Ichthyosis vulgaris skin shows normal loricrin staining in the granular layer (Hohl, 1993). Collectively, loricrin expression seems to be linked to an orthokeratotic phenotype of epidermal keratinization (Hohl, 1993). In lamellar ichthyosis with deficient expression of transglutaminase 1, both loricrin and involucrin are expressed but are not incorporated into CE (Hohl et al., 1993).

Loricrin is cross-linked and becomes a major constituent of CEs

Loricrin constitutes about 70% of the mass of CEs of foreskin epidermis and the amino acid compositions of isolated CE and loricrin are similar (Steven and Steinert, 1994; Steinert, 1995). It has been proposed that CE assembly is initiated with formation of a scaffold that consists of crosslinked soluble CE precursors including involucrin and cystatin A; other precursors including loricrin are incorporated later (Reichert et al., 1993; Steinert, 1995; Steinert and Marekov, 1995; Eckert et al., 1997) (Fig. 3). Intermolecular cross-links are formed between loricrin molecules or between loricrin and other CE precursor proteins such as involucrin, SPRRs, elafin, keratins, and filaggrin (Steinert and Marekov, 1995, 1997). Amino acid residues of human loricrin cross-linked by transglutaminases *in vivo* and *in vitro* include Lys4, Lys5, Lys88, Lys307, Lys315, Gln3, Gln6, Gln10, Gln153, Gln156, Gln215, Gln216, Gln219, Gln225, Gln305 and Gln306 (Candi et al., 1995; Steinert and Marekov, 1995, 1997) (Fig. 1). The preferential Gln/Lys residues used for crosslinkage differ between transglutaminases 1 and 3. Transglutaminase 1 establishes intermolecular cross-links to form large loricrin oligomers, while most of the cross-links formed by transglutaminase 3 involve intramolecular cross-links (Candi et al., 1995).

Loricrin mutations in Vohwinkel's syndrome (VS)

VS, also known as kerato(der)ma hereditaria mutilans, is a rare autosomal-dominant genetic skin disease (Vohwinkel, 1929). It is characterized by diffuse palmoplantar hyperkeratosis with a ''honeycomb'' appearance beginning early in life and by progressive formation of digital constricting bands (pseudoainhum) that are most frequently observed in the fifth fingers or toes. There is clinical heterogeneity among families. Transition from the hyperkeratotic palmoplantar lesions to normal skin is abrupt in some patients (Vohwinkel,

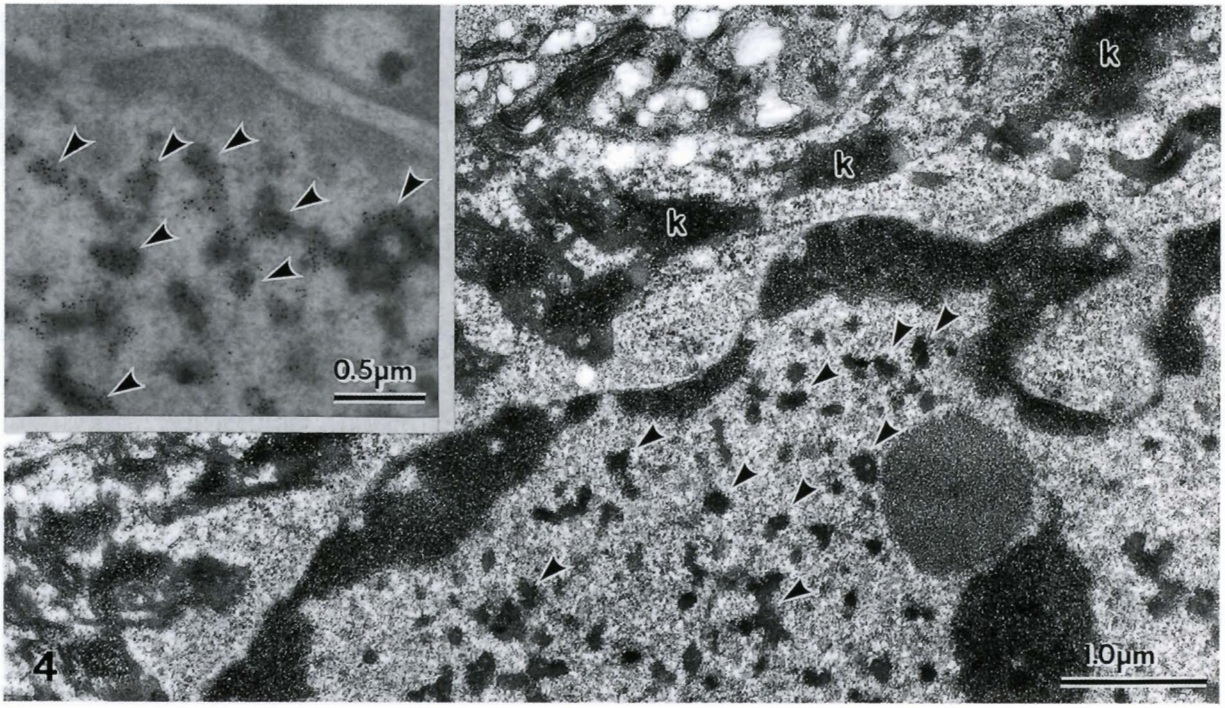

Fig. 4. Electron microscopy of Vohwinkel's syndrome. A granular cell contains many intranuclear granules (arrowheads) that are positively labeled with an antibody against the amino-terminus of loricrin, AF2340 (insert). k: keratohyalin granules. x 19, 600; insert: x 24,000

1929; McGibbon and Watson, 1977; Schamroth, 1986) while it is gradual in others (Cole et al., 1984; Korge et al., 1997). The patients originally described by Vohwinkel and by others (Gibbs and Frank, 1966; McGibbon and Watson, 1977; Chan Sing Pang et al., 1981; Schamroth, 1986) show hyperkeratotic papules or plaques on the elbows and knees as well as on the dorsa of hands and feet. These lesions have a peculiar, linear or starfish-shaped configuration. Some patients have ichthyosis on other parts of the skin (Camisa and Rossana, 1984; Cole et al., 1984; Camisa et al., 1988, Maestrini et al., 1996; Korge et al., 1997). Other associated symptoms include a high-tone acoustic impairment or deafness (Drummond, 1939; Gibbs and Frank, 1966; McGibbon and Watson, 1977; Chan Sing Pang et al., 1981), diffuse alopecia (Gibbs and Frank, 1966; Chan Sing Pang et al., 1981), transient plantar blistering (Gibbs and Frank, 1966), spastic paraplegia (Chan Sing Pang et al., 1981) and myopathy (Chan Sing Pang et al., 1981). Some patients show characteristic histopathological features (see below), while others do not (McGibbon and Watson, 1977; Chan Sing Pang et al., 1981; Cole et al., 1984; Schamroth, 1986).

We have examined members from two unrelated families with a unique subtype of VS (Maestrini et al., 1996; Korge et al., 1997). Some patients in both families had ichthyotic skin lesions but hearing impairment was not a feature. Both lesional and non-lesional skin showed characteristic morphological and immunohistochemical abnormalities. The epidermal cornified layer was thicker than normal with retained round nuclei and the granular cell layer was also thickened. Granular cells contained many intranuclear granules which were labeled with an amino-terminal loricrin antibody, AF2340 (a gift from Dr. D.R. Roop) (Figs. 3, 4). In addition, the expression of involucrin was increased. CEs were thinner than normal and sparsely labeled with loricrin-, but heavily with involucrin antibodies. Parakeratotic cornified cells contained loricrin-positive granules.

Molecular analyses using these two families have shown strong linkage to the EDC on chromosome 1q21. Sequencing of the loricrin gene has disclosed a heterozygous mutation; insertion of a G nucleotide (730insG) that shifts the reading frame in both families (Fig. 1). Consequently the carboxyl-terminal 84 amino acids are replaced by highly charged missense sequences that override the endogeneous termination codon extending the loricrin protein with an additional 22 amino acids.

However, it should be noted that loricrin has been excluded as a candidate gene in other families with VS (Korge et al., 1997; Akiyama et al., 1998), and therefore VS seems to be a genetically heterogeneous disorder.

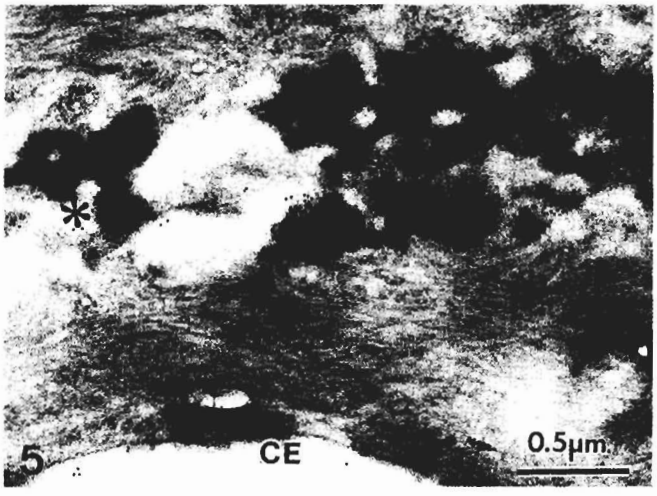

Fig. 5. Loricrin immunoelectron microscopy in progressive symmetric erythrokeratoderma. A parakeratotic cornified cell contains loricrin-positive aggregates (*) and shows very few labels on the cornified cell envelope (CE). x 28,800

Loricrin mutations in progressive symmetric erythrokeratoderma (PSEK)

Based on clinical, histological and immunohistochemical similarities to VS, we have identified another genetic disease of loricrin; specifically a type of PSEK (Ishida-Yamamoto et al., 1997), a rare systemic skin disease that is also inherited as an autosomal dominant trait. A hallmark of PSEK is widespread erythematous keratotic plaques. Clinical variations exist among different families, and even in the same family variations may exist among individuals. Some cases also show palmoplantar hyperkeratosis.

In one PSEK family the patients had features of palmoplantar keratoderma with a 'honeycomb' appearance and pseudoainhum (Ishida-Yamamoto et al., 1997). Histologically, there was marked hypergranulosis with parakeratosis in the epidermis. CEs were thinner than normal. Furthermore, loricrin was aggregated in the nucleus and CEs had less loricrin labeling when assessed by immunoelectron microscopy (Fig. 5). All these features were similar to those seen in skin biopsies in VS with loricrin mutations. Subsequent sequencing of the loricrin gene disclosed a heterozygous frame shift mutation, an insertion of a C nucleotide (709insC) (Fig. 1). This creates 91 missense amino acids at the carboxyl terminus and the 22-amino-acid extension similar to VS. This missense sequence is seven amino acids longer that that of the VS mutant protein. Despite the similarities in the mutations, the clinical differences between this PSEK family and the VS family with loricrin mutations might be related to the difference in length of the missense sequence, but this will need to be validated by identifying further patients with various loricrin mutations. Because the clinical and histological features are known to vary among different families with PSEK, it is likely that PSEK is also genetically heterogeneous.

Biological effects of the loricrin mutations

In the cases with loricrin mutations reported so far, the molecular pathology has involved frameshift mutations leading to delayed termination codons in the loricrin gene. The carboxyl-terminal domains of loricrin are replaced by highly charged sequences rich in arginine. Although the patients also express non-mutated loricrin transcribed from the normal allele, the majority of loricrin molecules are not incorporated into CE. The normal loricrin proteins are also aggregated within nuclei together with mutant loricrin, as was demonstrated by immunoelectron microscopy using a carboxyl terminal loricrin antibody that recognizes only normal loricrin (Fig. 3) (Korge et al., 1997). This may be interpreted as a dominant negative effect of the mutation. Although the exact molecular mechanisms for this finding are currently unknown, it could be related to the changes in the biophysical properties of the carboxyl terminus of loricrin (Ishida-Yamamoto et al., 1997). Since loricrin is expressed widely in the epidermis covering the entire body surface and the characteristic histological and immunohistochemical abnormalities are also noted in the un-involved skin of VS patients, it is curious that the skin lesions are rather confined to certain sites. Conceivably, there might be some redundancy in the structure of CE or other CE precursor proteins might be compensating for the abnormal loricrin in uninvolved areas (Ishida-Yamamoto et al., 1997).

Although the exact etiological significance is not clear, nuclear accumulation of loricrin is characteristic to VS and PSEK involving loricrin mutations. Aggregation of loricrin or L-granules is normally seen in newborn mice epidermis and is rarely seen in normal human skin (see above), but these are not confined to the nucleus. Loricrin is a small molecule and could possibly be translocated through nuclear pores by passive diffusion into the nucleus (Nigg, 1997). In the patients with loricrin mutations the mutant loricrin molecules are seen condensed in the nucleus. Wild type loricrin molecules are rather diffusely distributed both in the cytoplasm and in the karyoplasm (Korge et al., 1997). The abnormal distribution of mutant loricrin could be the direct consequence of the frameshift mutation, as the resultant arginine-rich sequence in the carboxyl terminal peptide includes bipartite signal motifs which may act as a nuclear targeting sequence (Korge et al., 1997; Nigg, 1997).

Loricrin knockout shows redundancy of CE precursor proteins

Recently, loricrin knockout mice have been developed that show congenital erythroderma with a shiny, translucent skin and low birth weight (de Viragh et al., 1997). The skin of these mice, however, healed spontaneously within 4-5 days forming an apparently normal epidermis thereafter. Although defects in epidermal barrier function remained in the older mice, the recovery to a normal appearance suggests some functional redundancy among CE components or possible compensatory upregulation of other structural components of CEs including involucrin and SPRR. To date, no human disorder has been attributed to a functional knockout of loricrin.

Conclusions and clinical implications

We have demonstrated that certain variant forms of VS and PSEK result from mutations in the loricrin gene. Because of the distinctive but homologous nature of clinical phenotypes and molecular pathology, it seems appropriate to refer to these disorders collectively as "loricrin keratodermas". The discovery of specific loricrin mutations and appreciation of the clinical consequences make it clear that the correct formation of CE is essential for normal skin biology. We have to wait, however, until more patients with loricrin mutations are disclosed before clear genotype-phenotype correlations emerge and a more complete picture of loricrin pathophysiology is established. To address the exact mechanism by which the loricrin mutations cause the clinical sequelae, further analysis involving cell biological and transgenic animal studies with various loricrin mutations as well as biochemical and physiological analyses of the patients' CEs will be required.

Identification of the genetic basis of VS and PSEK allows us to seek better treatments for the patients. The patients with loricrin mutations described above responded well to systemic retinoid therapy. Because retinoic acid suppresses the expression of both wild type and mutant loricrin in the presence of an apparent redundancy of CE precursor proteins, it is not surprising that retinoids improve loricrin keratodermas manifesting dominant negative effects of the mutated loricrin. It would be desirable, however, to develop drugs which specifically suppress loricrin expression with less side effects than retinoic acid, since retinoids also inhibit the expression of other CE precursor proteins that may be potentially involved in biological compensation. Finally, the detection of the precise nature of the mutation in each family is a prerequisite for consideration of prenatal diagnosis as well as for development of gene therapy against these genetic skin diseases in the future.

Acknowledgements. We thank Dr. J.A. McGrath for critical reading of the manuscript. The original studies by the authors were supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan.

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