A functional “knockout” of human keratin 14

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The importance of keratins and other intermediate filaments in the maintenance of tissue structure is emphasized by the discovery that many hereditary skin-blistering diseases are caused by mutations in keratin genes. Here, we describe a situation in which keratin 14 (K14) is missing altogether in the epidermis: A homozygous 2-nucleotide deletion in exon I of the K14 gene causes premature termination of the mRNA transcripts upstream from the start of the rod domain and results in a K14 null phenotype. In this individual no keratin intermediate filaments are visible in basal epidermal cells, although filaments are present in the upper layers of the epidermis. No compensating keratin expression is detected in vivo, and K14 mRNA is down-regulated. The individual, diagnosed as Köbner (generalized) EBS, suffers from severe widespread keratinocyte fragility and blistering at many body sites, but although the phenotype is severe, it is not lethal. This K14^{-/-} phenotype confirms that only one K14 gene is expressed in human epidermis and provides an important model system for examining the interdependence of different keratin filament systems and their associated structures in the skin.

[*Key Words:* Keratin; K14 null; EBS; skin disease; deletion mutation; intermediate filaments]

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Keratins constitute >80% of the total cell protein in the differentiated keratinocyte of the skin. The coexpressed keratin pair K5 and K14 are the primary keratins of stratifying epithelia and major structural proteins in the mammalian body. Epidermal keratinocytes synthesize large amounts of keratins K5 and K14 in the basal proliferative layer but switch to synthesis of secondary keratins K1 and K10 as the cells leave the basement membrane and begin their progressive terminal differentiation toward the surface. Keratins are long-lived, and immunodetectable K5 and K14 persist throughout keratinocyte transit in the epidermis (normally ~7 days). Transfection experiments in cultured cells suggest that a scaffold of K5 and K14 may be a prerequisite for correct assembly of K1 and K10 (Kartasova et al. 1993). K5 and K14 are also expressed in a variety of other tissues, including all stratified squamous epithelia in the alimentary canal and urogenital tract, and the basal cells of glandular and ductal epithelia.

In the epidermolysis bullosa simplex (EBS) diseases, the basal layer of the epidermis and the mucosal epithelia are fragile. These diseases involve mutations in K5 and/or K14. Keratins are heteropolymers, and filaments are composed of a type I keratin protein and its specific type II partner; mutations in key domains of either of the two contributing genes result in similar phenotypes

(Lane 1994). These mutations lead to localized basal cell rupture and epidermal blistering on physical trauma to the skin. The severity of the EBS diseases, from thickened and fragile palm and sole skin to widespread and sometimes fatal blistering, is correlated partially with the location of the defect in the protein structure (Rugg et al. 1993b; Lane 1994). The fact that keratins form polymeric structures built up from heterodimers explains the dominant negative phenotype of these mutations, and it has been reported that very small amounts (<1%) of defective protein can “poison” a whole cytoskeleton system and compromise its physical resilience (Coulombe et al. 1990). However, all of the spontaneous pathogenic mutations described to date still leave the keratinocytes with at least some keratin filaments.

The most severe of these diseases, the Dowling-Meara form of EBS, is characterized by microscopically visible aggregates of keratins in the cytoplasm of basal keratinocytes. In the absence of a null mutation it has been argued that these keratin aggregates might cause more damage than the loss of a filament network. Although much information on these diseases has come from transgenic mouse experiments (Vassar et al. 1991; Cheng et al. 1992), there is no information yet on the effect of a deletion or “knockout” of the epidermal keratin genes. However, transgenic ablation in mice of the gene for K8,

which is expressed in the early embryo, is nearly always lethal at mid-gestation (Baribault et al. 1993).

Here, we report a spontaneous loss of K14 expression seen in a child, the offspring of a consanguineous marriage, who carries two copies of a deletion mutation in K14, leading to loss of K14 expression in the skin and other tissues of the body. In this individual no keratin intermediate filaments (IFs) are seen in the basal cells of the epidermis. The phenotype is severe but not lethal.

Results

Clinical phenotype

A child with severe generalized epidermal blistering was diagnosed at 20 months as suffering from Köbner-type EBS (Fig. 1). Blisters were first seen at 3 days after birth when they occurred virtually anywhere on the body, including the arms, legs, trunk, face, scalp, and oral mucosa, apparently without cause or upon mild friction. When the patient was examined at the age of 5 years the skin was still fragile and blisters were evident, mainly on the hands, feet, and neck. He was able to eat normally. There was no thickening of the palms and soles. The hair was normal; the front teeth were discolored and notched but not pitted; fingernails were ridged and of uneven thickness. The parents reported that the tendency to blister had possibly diminished with age; healing blisters left areas of hypopigmentation. Neither of the parents, who are first cousins, is affected by blistering and there were no reports of skin fragility or blistering disorders in any other family member.

The most conspicuous abnormality of the epidermis visible by light microscopy was the relative pallor of the lowest cell layers of the epidermis (Fig. 2). Diagnostic

Figure 1. Phenotype of the K14^{-/-} child. Hands and wrists show the remains of superficial blisters, slight loss of pigmentation where previous blisters have healed, and nail dystrophy. Blisters were found all over the body; the widespread distribution of blisters would classify this patient as Köbner-type EBS, but the severity of this case is similar to that usually associated with the Dowling-Meara form of EBS.

Figure 2. Light micrograph of skin biopsy showing epidermal changes including reduced staining in the cytoplasm of basal and some suprabasal cells. Rubbed skin shows a low-level split in the basal layer (arrowhead) of the epidermis. The rest of the epidermis appears normal. Semithin section stained with methylene blue and azure II; Bar, 50 μ m.

electron microscopic examination identified an intra-epidermal fracture plane within the basal keratinocytes, very close to the basal cell membrane and, therefore, confirmed the defect as a form of EBS (Fig. 6a, below). An immunohistochemical screen of skin sections gave an unusual negative result with an antibody to K14 (Fig. 3), suggesting a defect in this major epidermal protein.

Homozygosity at the K14 locus

In view of the apparent absence of K14 and the consanguinity in the family suggestive of recessive inheritance, the patient's DNA was examined at the *KRT14* locus on chromosome 17q. Genomic DNA was extracted from blood samples of family members, and individuals in the EBS-Köbner family were genotyped with four polymorphic gene probes (Gyapay et al. 1994; McLean et al. 1994) located on chromosome 17 that are known to be physically close to the K14 gene. The affected child was found to have inherited the same haplotype from both parents and, therefore, is homozygous at the K14 locus (Fig. 4). The probes flank the K14 gene, and their close physical proximity means that the probability of recombination between the probes and the K14 gene is negligible. Taken with the fact that the parents are first cousins, these results are consistent with the homozygous inheritance of a recessive K14 mutation in the affected individual and suggest that the heterozygous state may be asymptomatic.

A 2-nucleotide deletion in exon I of K14

Keratin mRNA was extracted and reverse transcribed from keratinocytes cultured from the patient's epidermis. Sequencing of the complete cDNA for K14 revealed the deletion of nucleotides 313 and 314 from both alleles of the K14 gene (Fig. 5a,b). The unaffected parents were found to be heterozygous for this defect, consistent with a recessive inheritance pattern. PCR analysis of 50 unrelated, unaffected individuals detected no similar dele-

Figure 3. Immunofluorescence staining of frozen acetone-fixed sections with monoclonal antibodies to keratins reveals the absence of K14 expression in the skin. K5 staining gives a similar picture. (a) LL001 (to K14) staining the basal layer of epidermal keratinocytes in a normal skin section; (b) no LL001 staining of the EBS patient's skin. Solid line indicates position of basement membrane. (c) Similar section of the patient's skin stained with AE14 (K5): Note the concentration of staining along the basal cell membrane and around the nucleus. (B) Forming blister cavity where basal epidermal cells have ruptured; (E) epidermis; (D) dermis. Magnification, 347 $\times$.

tions, making it unlikely that this defect represents a common polymorphism in the general population (Fig. 5c).

The 2-nucleotide deletion produces a frameshift leading to a premature stop codon in the third codon downstream from the mutation and is predicted to result in a severely truncated form of K14 (Fig. 5d), consisting of the first 104 amino acids of the nonhelical head region followed only by two tryptophan residues.

Absence of keratin filaments from basal cells in skin

To study the effects of this severe truncation of K14, skin samples were examined more closely by light and electron microscopy.

Monoclonal and polyclonal antibodies to keratins

were used on frozen sections of skin to assess the full range of keratins expressed in this abnormal epidermis. In control skin samples, cells in the basal keratinocyte

Figure 4. Homozygosity of the *KRT14* locus in this family. (a) Parents and son in the family studied here were genotyped with polymorphic markers within and flanking the type I keratin gene cluster on human chromosome 17q. The EBS-affected proband (arrow) has inherited the same haplotype from each parent in this region and therefore is homozygous at the K14 locus. These results are consistent with homozygous inheritance of a recessive K14 mutation through the consanguineous pedigree. (b) Genetic map of the type I keratin locus on chromosome 17q. All probes used were Génethon microsatellites except K10V2 (an intragenic polymorphism of K10). Marker D17S800 and the K10 and K14 genes are physically close (within ~ 100 kb: W.H.I. McLean, unpubl.). Distances in centiMorgans are taken from the 1993–1994 Genethon map (Gyapay et al. 1994).

Figure 5. The DNA sequence and predicted amino acid sequence obtained from clones containing part of the region encoding the head domain of K14 from a normal K14 allele of an unaffected individual (a) and the EBS-affected child (b). Sequences were consistent in several different clones. A representative sample of radiolabeled PCR fragments spanning the deletion and analyzed on a 6% denaturing gel is shown in (c). Lanes 1–4 and 6–10 are from genomic DNA from unaffected individuals (K14+/+), the affected child (K14-/-), and the unaffected parents (K14+/-). Lane 5 shows the products of a similar analysis of cDNA from the affected child. (d) Diagram showing the position of the 106-amino-acid peptide (solid line) predicted to be synthesized from the mutated message (arrow shows position of premature stop codon). The rest of the K14 protein is shown in dotted lines; the remainder of head, rod domain, and tail domain are untranslated.

layer are strongly stained by monoclonal antibodies LL001 (to K14) and AE14 (to K5) and RbaK5 antiserum (Purkis et al. 1990). In contrast, there was a complete absence of K14 staining in the patient's basal cells (see Fig. 3). Traces of K5 staining were observed along the basal cell membrane and around the nucleus of intact cells. Antibodies to K10 (mAb LHP1) and K1 (anti-peptide serum RbaK1) showed an apparently normal distribution of these keratins in the suprabasal layers (data not shown). K17 staining of the basal cells was seen using the mAb E3 (not shown); staining was similar to that seen with K5 antibodies. K17 staining in basal cells is seen occasionally in skin. Some suprabasal cells were also positive for K16 (mAb LL025), which is characteristic of stressed epidermis. As expected, all keratinocytes were negative for K8 (mAb LE41) and K19 (mAb LP2K).

Closer examination of both fractured and intact basal keratinocytes by electron microscopy revealed the complete lack of normal keratin IFs ("tonofilaments"), despite the presence of other normal keratinocyte cytoplasmic constituents, including mitochondria, Golgi apparatus, melanosomes, and rough endoplasmic reticulum (Fig. 6b). Keratin filaments were present, although sparse, in cells of the first suprabasal layer but were denser by the mid-spinous layer, where they appeared shorter than normal and in small clumps or aggregates (Fig. 6c). No abnormalities of tonofilaments or other cellular constituents were identified in the upper spinous, granular, or cornified layers.

Hemidesmosomes appeared quantitatively normal but were not always fully developed (Fig. 6b). Whereas the outer or membrane-associated plaques of the hemidesmosomes looked normal, the inner (cytoplasmic) plaques were sometimes rudimentary. The inner plaque is the part of the hemidesmosome normally associated with the tonofilaments. Although no tonofilaments of normal thickness were identified, a few thin short poorly formed filaments, about the same thickness as microfilaments, were noted in the peripheral cytoplasm of the basal cells where the hemidesmosomes occurred.

In the keratinocytes lacking tonofilaments, desmosomes were reduced in number and were of variable size. Otherwise, they were structurally normal with well-developed electron-dense plaques but lacking normal filamentous connections. At the apical side of some cells, and in the first suprabasal layer, desmosomes were associated with small amounts of amorphous material, possibly composed of keratin (Fig. 6d).

In the mid-spinous layer desmosomes were more numerous and associated with filaments and, more often, small tonofilament aggregates (Fig. 6c). Despite the structural abnormalities of desmosomes and hemidesmosomes, the basal cells remained adherent to the basement membrane and to each other. Some widening of the lower intercellular spaces of the epidermis was evident, emphasizing numerous cell processes or lamellipodia that would normally be tightly packed and less conspicuous. The epidermis of the patient's father (not shown) was ultrastructurally normal with well-developed tonofilaments throughout all cell layers.

Figure 6. Ultrastructure of K14^{-/-} epidermal cells. (a) Appearance after mild friction. A fracture (asterisks) is evident in the lower part of the basal layer. The largely intact epidermis (E) is above the split, whereas fragments of basal cells (arrows) remain attached to the basement membrane and the underlying dermis (D). Bar, 10 μ m. (b) Basal keratinocytes adjacent to the fracture are devoid of tonofilaments, but other cell constituents including nuclei (n), mitochondria (mi), and melanosomes (me) are normal. Hemidesmosomes (hd) show electron-dense membrane-associated plaques. Bar, 1 μ m. (c) Keratinocyte in the mid-spinous region of the epidermis, showing filamentous material present in short bundles close to the nucleus (n) and also in larger tonofilament aggregates (ta) more peripherally in the cell, associated with desmosomes (ds). Bar, 1 μ m. (d): Desmosomes (ds) between two adjacent basal keratinocytes (nuclei n¹ and n²) are ultrastructurally normal. Electron-dense material is closely associated with the desmosome plaques, but normal tonofilaments are absent. (g) Golgi apparatus; (dt) melanocyte process containing four melanosomes. Bar = 1 μ m.

Keratin filaments are present in cultured cells

Keratinocytes are known to modify their keratin expression pattern on transfer to a simple tissue culture system (Weiss et al. 1984), where they behave as if they are in a constant state of wound healing. In normal cells the primary keratins K5 and K14 persist but K1 and K10 are down-regulated, and the secondary keratins K6 and K16 (and/or K17), typically up-regulated in wound healing, become prominent. K19 is also up-regulated to a variable degree, and in long-term culture simple epithelial primary keratins, K8 and K18, also become detectable.

Epidermal cells cultured from the affected patient were stained with a number of keratin antibodies and examined by immunofluorescence microscopy. In the cultured cells, long sinuous filaments were seen on staining for K17, and K5 staining also appeared to be filamentous (Fig. 7). However, there was no suggestion of

Figure 7. Immunofluorescence staining of patient's epidermal keratinocytes in tissue culture, stained with monoclonal antibodies E3 against K17 (a) and RCK102 recognizing K5 and K8 (b). Both antibodies show well-developed filamentous networks, with desmosomes (arrow), regardless of the absence of K14. The lack of reaction with K8-monospecific antibody LE41 both in culture and on parallel immunoblots (not shown) indicates that RCK102 is reacting with K5 in the epidermal cells. (n) Nuclei of individual cells. (a) Magnification, 1364 $\times$; (b) magnification, 868 $\times$.

concentration of K5 around the cell periphery as there had been in the blistered epidermis. Positive filamentous immunostaining was also observed with monoclonal antibodies to K6 (mAb KA12), K16 (mAb LL025), and K8/K7 (mAb CAM 5.2). As in the tissue sections, there was a complete absence of K14 staining.

Keratin expression in the absence of K14

When tissue samples and cultured keratinocytes from the affected individual were analyzed by SDS-PAGE and immunoblotting, no K14 immunoreactivity was observed in the cytoskeletal extract (Fig. 8a) or total cell extract. India ink staining of the same extracts revealed an absence of protein in the expected position (Fig. 8b). K5 was present in the cultured keratinocytes in near-normal amounts, as was K16 and K17 (Fig. 8a). K6 and K7 were also present (not shown). There was no detectable K8, K18, or K19 in second-passage keratinocytes. Similar analysis of total tissue extract from the skin biopsy confirmed the presence of K5 in this sample. Antibody RCK102 stained a single band corresponding to K5, and similar staining was seen with AE14, although this was much weaker (Fig. 8c). Again, no K14 staining was seen using LL001. As expected in epidermis, large amounts of K10 (using LHP1) were present. K17 (with E3) was barely detectable. No staining was observed at the expected molecular weight with a monoclonal antibody to K16 (LL025), although there appeared to be a weak cross-reactivity of this antibody with K10.

K14 mRNA is undetectable by in situ hybridization

Nonisotopic in situ hybridization studies using digoxigenin were carried out on skin sections from the affected child and a healthy individual (Fig. 9). The distribution of K1, K5, and K10 mRNA was similar between the two samples. Using the K5 probe, labeling was seen in the basal layer of epidermis, and, to a much lesser degree, in the suprabasal layers. A strong signal in the suprabasal layers was seen with probes to K1 and K10. The K14 probe labeled sections from the healthy individual with a distribution similar to that seen for K5. However, no

Figure 8. Immunoblotting demonstration of keratins present in test and control keratinocytes. (a) Blots of cytoskeletal extracts from primary keratinocyte cultures, from the affected child (EBS) and an unaffected individual (Normal), using monoclonal antibodies AE14 to K5, LL001 to K14, LL025 to K16, and E3 to K17. (b) India ink-stained blot of the same two extracts as in a, plus a cytoskeletal extract from TR146 cells and a total cell extract of the patient's cultured keratinocytes. Note the absence of the K14 band in extracts from the patient's cells. (c) Immunoblots of total skin protein from the patient stained with monoclonal antibodies LHP1 (to K10), RCK102 (to K5 and K8), AE14, E3, LL025, and LHP1. Although there appears to be no K17 in this sample, close examination of the blot indicated that there was a faint immunoreactive band at the expected molecular weight. Lack of material prevented analysis with a larger sample to confirm the presence of this protein.

Figure 9. In situ hybridization with antisense RNA probes to keratins K5 (*a,b*), K14 (*c,d*), K1 (*e,f*) and K10 (*g,h*) on cryostat sections using digoxigenin. (*a,c,e,g*) Skin from affected child; (*b,d,f,h*) control healthy skin. The signal is a blue product of alkaline phosphatase, NBT, and X-phosphate. Slides were photographed without counterstaining. The lack of K14 signal in the affected child is shown in *c*. (The trace of basal dark coloration on this black-and-white photograph is the normal melanin in the basal keratinocytes and is clearly distinguishable from the blue mRNA reaction product in the section itself.) Magnification, 240 $\times$.

specific signal for K14 mRNA could be detected in the K14 $-/-$ tissue sections.

Similar results were obtained using isotopic in situ hybridization. Both K5 and K14 antisense cRNA probes displayed accumulation of signal over the basal layer of normal epidermis, whereas suprabasal layers showed a markedly lower amount of silver grains that were within or slightly above the level of background, as determined with sense cRNA probes used as controls. When K5 and K14 probes were hybridized to skin sections from the affected child, the K5 probe gave a strong specific signal, whereas the K14 probe only yielded silver grains at the background level and was considered negative (not shown). However, mRNA for K14 was successfully amplified from cultured keratinocytes and used to generate the K14 cDNA for sequencing; therefore, some K14 mRNA is clearly present. Semiquantitative PCR titra-

tions suggest that this <10% of the control level of mRNA for K14 (data not shown).

Discussion

Keratins are obligate heteropolymers composed of type I and type II IF proteins. Polymerization proceeds by the formation of type I–type II heterodimers (Hatzfeld and Weber 1990; Steinert 1990), followed by a cascade of intermediate structures that assemble to form 10-nm filaments. The highly conserved boundary motifs of the central rod domain appear to be essential for correct filament assembly (Albers and Fuchs 1987, 1989; Hatzfeld and Weber 1991; Letai et al. 1992), and missense mutations in these regions result in filament aggregation and the most severe dominant skin fragility syndromes such as Dowling–Meara EBS (Coulombe et al. 1991; Lane et al. 1992; Stephens et al. 1993). Mutations occurring between these conserved end domains, including a codon deletion, appear to result in milder phenotypes (Chan et al. 1993; Chen et al. 1993; Humphries et al. 1993; Rugg et al. 1993a). Here, we describe ablation of K14 producing a severe clinical phenotype. The fragility of the epidermis resulting from this functional knockout of K14 is similar in severity to Dowling–Meara EBS, although it is ultrastructurally distinct, and the patient was classified as Köbner-type EBS from the body distribution of the blisters. The widespread distribution of the nonclustered blisters and the lack of ultrastructurally visible keratin aggregates characterize Köbner EBS as a distinct disease, and the frequency of this specific phenotype is probably very rare. The similarity of Dowling–Meara EBS to the null phenotype described here suggests that mutations in the helix boundary peptides render the keratin cytoskeleton ineffective. K5 and K14 are expressed in tissues other than epidermis, such as oral epithelium. Although lesions of the oral mucosa were seen in this patient, as with many other Dowling–Meara EBS patients, these oral lesions were not a major aspect of the disease. The differential involvement of different tissues might be attributable to different physical stresses in these sites and/or expression of compensatory factors.

Keratin end domains are targets for deletions

The deletion reported here occurs within a quasirepeat of a 10-bp sequence located just upstream of the predicted stop codon and is consistent with a modified mispairing model of deletion (Cooper and Krawczak 1993). Similar mutational mechanisms have been seen in the CFTR gene, the AT3 gene, and others (Cooper and Krawczak 1993). The subdomains V1 (in the head domain) and V2 (in the tail domain) of keratins consist largely of glycine-rich crude repeats and, therefore, the DNA sequences encoding these domains are potential targets for deletion–insertion mutations. In-frame insertional polymorphism of the V1 and V2 subdomains of a number of keratins has been described (Korge et al. 1992a, 1992b) and produces no known pathology. As seen here, a frameshift deletion in the V1 domain can produce a functionally

null allele. Similar frameshifts might be responsible for some other reported cases of severe recessive EBS-like skin-blistering disorders or other EBS cases where keratin filaments have been reported to be sparse (Ito et al. 1991). Deletions in these domains are a common feature of keratin pseudogenes, further demonstrating their mutational sensitivity (Savtchenko et al. 1988; Troyanovsky et al. 1992).

Basal cells are devoid of filamentous keratin

Filaments were not observed in the basal keratinocytes and, therefore, it appears that there is no compensatory expression of a type I keratin in these cells that is able to form filaments with K5 (Figs. 2, 3, and 6). The presence of K5 staining at the basal membrane may indicate the stabilization of this protein by an alternative route, possibly by interaction of the keratin with desmosomal and hemidesmosomal proteins. A similar phenomenon is seen in embryonic stem cells expressing a dominant-negative mutation that eliminates K18 function (Trevor 1990; Baribault and Oshima 1991) and in K8 knockout mice (Baribault et al. 1993). It has also been suggested that there is an interaction between IF networks and the nuclear membrane (Georgatos and Blobel, 1987; Georgatos et al. 1987; Worman et al. 1988). The apparent perinuclear localization of K5 staining suggests a protein in the nuclear membrane capable of stabilizing K5. The colocalization of K5 and K17 staining in the epidermis may indicate that K5 is partially assembling with K17 but that this complex is unable to progress to 10-nm filaments. The thin filamentous material seen in the peripheral cytoplasm of the basal cells could be protofilaments of K5/K17, although we have no direct evidence for this.

Keratin filaments form in cultured cells

K5 formed filaments in the K14^{-/-} cultured keratinocytes (Fig. 7). These cells, unlike the basal keratinocytes of the epidermis, express both K16 and K17. It is possible that K5 forms a network with K16 in these cells or that it is incorporated into a preexisting K6/K16 cytoskeleton. Likewise, K17 is also able to form filaments in K14^{-/-} cultured keratinocytes. It is not known which type II keratin is the normal partner for K17. The keratinocyte cell line TR146 forms well-developed K17-containing networks. These cells express a number of keratins, including K6, but do not express significant amounts of K16 (Purkis et al. 1990). K6 and K17 may represent natural copolymers, as K17 expression often (but not always) parallels K6 and K16 expression in vivo. K6 is present in the cultured K14^{-/-} cells but not in the basal cells of the epidermis, and this may account for the difference between the two systems. Alternatively, as only very small amounts of K17 were found in the epidermis, a failure to form filaments with K5 may be attributable to K17 being present below a critical concentration. In vivo polymerization studies and/or transfection experiments, will be required to address directly these issues.

K1 and K10 can polymerize without K14

By electron microscopy we were unable to detect IFs in the cytoplasm of the basal keratinocytes, although filament bundles were clearly present in suprabasal cells. It has been suggested that the presence of a K5/K14 cytoskeleton is a prerequisite for the formation of a normal K1/K10 network in living cells (Kartasova et al. 1993). In the case reported here, filaments of K1/K10 keratins in the suprabasal cells did show some abnormalities. Filaments were sparse in the epibasal layer. In the mid-spinous layer, where more filamentous material was present, there was a tendency to form clumps (Fig. 6c). The clumping bore a superficial resemblance to disturbances of filament networks seen in keratinocyte fragility syndromes involving mutations in K1 and/or K10 (Ishida-Yamamoto et al. 1992). This suggests that the absence of a K5/K14 cytoskeleton has some deleterious effect on the K1/K10 network. No suprabasal cytolysis was ever seen in the samples examined here, possibly because the total lack of filaments in the basal cells renders them the weakest point in the system.

No detectable interference from K14 head fragment

Short peptides that mimic important sequence motifs have been shown to interfere with polymerization of normal keratins (Kouklis et al. 1991; Hatzfeld and Weber 1992; Herrmann et al. 1992). The presence of mutant K14 mRNA in the cultured keratinocytes, along with at least one or two related type I keratins, raised the possibility that the short fragment translatable from the mutant K14 mRNA might provide a natural competition experiment. However, filamentous K17 looked completely normal in these cells (Fig. 7a). Analysis of total cell protein on high-resolution polyacrylamide gels (Schägger and von Jagow 1987) failed to reveal any fragments in an appropriate molecular weight range for derivation from this message (not shown). Although the scarcity of material precluded pulse-chase experiments, it seems likely that this fragment would have been proteolyzed rapidly, as this is known to be the fate of unassembled whole keratin monomers (Domenjoud et al. 1988; Kulesh et al. 1989; Lu and Lane 1990).

Instability of mutant K14 mRNA in vivo

Compared with controls, a reduction in the amount of K14 mRNA levels is evident in the K14^{-/-} cells both in the epidermis in vivo and in the cultured keratinocytes. There are several reports of frameshift and nonsense mutations resulting in reductions of steady-state levels of mRNA (Cooper and Krawczak 1993 and references therein). The amount of mRNA appears to be correlated with the position of the mutation in the gene; mutations near the 5' end of mRNA appear to result in lower levels of message than those toward the 3' end. The mechanism by which this occurs is not understood but may result from the failure of incompletely translated mRNA to be protected from RNase digestion. The

failure to detect mRNA in tissue sections is consistent with these reports. However, K14 mRNA was detectable by PCR in cultured keratinocytes from this individual; semiquantitative PCR suggested that it was <10% of that found normal keratinocytes, and the amount would appear to be below the detection level of the *in situ* hybridization methods used here.

Single-copy expression of K14 is sufficient for normal skin function

Our observations support the suggestion that there is only a single gene for K14 expressed in human epidermis. Electrophoretic analysis of a total protein extract from cultured keratinocytes failed to identify the predicted head fragment of K14, suggesting that it is proteolyzed rapidly. Both parents are heterozygous for this mutation and have no abnormal phenotype, indicating that the predicted head fragment of K14, if present, does not affect filament integrity. More important, these results indicate that the product of one K14 gene is sufficient to produce a fully functional K5/K14 cytoskeleton.

The disorder described here has the potential for gene therapy, as the introduction of a single copy of the normal K14 gene should restore a normal filament network. This strategy has been applied successfully to the treatment of autosomal recessive adenine deaminase deficiency (Miller 1992) and familial hypercholesterolemia (Grossman et al. 1994) and is undergoing trials for other monogenic recessive diseases. Long-term clinical implications for dominant keratin disorders are that gene therapy based on the knockout of a deleterious mutant gene will be sufficient to restore the epidermis to normal function, as seen here from the heterozygous K14+/- parents.

Materials and methods

Sources of material

Skin biopsy specimens were taken from the patient and his father, under local anesthesia with informed consent, using a "shave biopsy" method that removes small pieces of epidermis and superficial dermis. The samples were from clinically uninvolved skin on the patient's upper thigh and from the father's upper arm. Another biopsy specimen was taken from the patient's thigh by rubbing the skin gently with a pencil eraser. Blood samples were taken from the patient and both parents for extraction of DNA. For tissue culture, keratinocytes were isolated using the shave biopsy method from the patient in an area of intact skin. Cells were cultured according to Rheinwald and Green (1975).

Light and electron microscopy

Samples were fixed in half-strength Karnovsky fixative for 4 hr at room temperature, postfixed in 1.3% osmium tetroxide in distilled water, and dehydrated and embedded in epoxy resin as described previously (Eady 1985). Semithin resin sections (1 μ m thick) for light microscopy were cut on a Reichert OMU4 ultramicrotome and stained with azure II and methylene blue. Ultrathin sections (80–100 nm thick) were cut on the same

microtome, stained with uranyl acetate and lead citrate, and examined in a JEOL 100CX electron microscope operating at 80 kV.

Indirect immunofluorescence microscopy

The specimens were embedded in O.C.T. and snap-frozen in isopentane cooled with liquid nitrogen. Sections, 5 μ m thick, were cut on a Bright's cryostat (Bright Instrument Co Ltd., Huntington, UK) and collected on gelatin and chrome alum subbed slides and allowed to dry for 10 min. Sections were washed in PBS and incubated with normal rabbit or swine serum. Antibody incubations were for 1 hr at room temperature in a humid atmosphere according to methods described by Kennedy et al. (1985). Sections were incubated with monoclonal or polyclonal antibodies, washed well with PBS, incubated in a second antibody, washed again, and mounted in 0.1% *p*-phenylenediamine in glycerol/PBS. Plates or flasks of cultured keratinocytes were washed briefly with PBS, fixed in 1:1 methanol/acetone for 5 min, air-dried and spotted with antibodies in a grid of small drops, and processed as for tissue sections. Monoclonal antibodies used were LL001 to K14 (Purkis et al. 1990), RCK102 to K5 and K8 (Broers et al. 1986), LE41 to K8 (Lane 1982), CAM5.2 to K8 and K7 (Makin et al. 1984), LP2K to K19 (Stasiak et al. 1989), AE14 to K5 (Moll et al. 1988), LL025 to K16 (Wetzels et al. 1991), E3 to K17 (Guelstein et al. 1988), KA12 to K6 (R. Nagle, pers. comm.), and LHP1 to K10 (Leigh et al. 1993). A polyclonal antiserum to the carboxy-terminal peptide of K5 (RbaK5; Purkis et al. 1990) and another to the carboxy-terminal peptide of K1 (RbaK1; Leigh et al. 1993) were also used. Second antibodies used were rabbit anti-mouse immunoglobulins and swine anti-rabbit immunoglobulins conjugated with FITC (DAKO), diluted 1:200 in PBS/1% BSA for tissue staining, and sheep anti-mouse immunoglobulin conjugated to FITC (Sigma), diluted 1:50 in expired tissue culture medium/10% fetal calf serum, for cell staining. Negative controls included incubations in the presence of the second antibody alone and with an inappropriate primary antibody.

Immunoblotting

Keratins were extracted from primary cultures of keratinocytes obtained from the affected child, from a healthy individual, and from an established keratinocyte cell line, TR146 (Rupniak et al. 1985). The cytokeratins were analyzed by SDS-PAGE followed by immunoblotting as described previously (Lane et al. 1992). Samples containing total cell extracts were obtained by solubilizing the cells or tissue directly in SDS sample buffer. Antibodies tested were PCK26 to K1, K5, K6, and K8 (Sigma/Biomakor), E3 to K17, α FA to most IFs (Pruss et al. 1981; Cooper et al. 1984), AE14 to K5, LE41 to K8, LHP1 and LH2 to K10 (Leigh et al. 1993), LL001 to K14, LL025 to K16, LP1K to K7 (Lane et al. 1985), KA12 to K6 (R. Nagle, pers. comm.), LCN18 to K18 (E.B. Lane, unpubl.), LP2K to K19 (Stasiak et al. 1989), and RCK102 to K5 and K8 (Broers et al. 1986).

Linkage analysis

Individuals from the EBS-Köbner kindred were genotyped with the following polymorphic DNA probes. (1) *D17S800*, *D17S791*, and *D17S798* (Généthon): These CA-repeat sequence were PCR amplified from genomic DNA with a ³²P-labeled primer and analyzed on 6% denaturing gels according to the recommended protocol in the Généthon Catalogue (Gyapay et al. 1994). (2) *K10V2* polymorphism: This variable number tandem repeat (VNTR) polymorphism in the V2 domain of the K10 gene was

PCR amplified and analyzed on 3% NuSieve-1× TBE minigels according to the modified protocol (McLean et al. 1994).

Sequence analysis

Poly(A)⁺ mRNA (QuickPrep Micro mRNA purification Kit, Pharmacia), extracted from keratinocyte cultures, was reverse transcribed with oligo(dT) (12–18 base), and two fragments of K14 cDNA (257–759 and 496–4808, EMBL accession number J00124) were amplified by PCR using the following primers: 5'-GGGGGAGCCTATGGGTGGGG-3' (496–516); 5'-AGT-GCTTGGGCAGGAGAGGGG-3' (4788–4808); 5'-TACCCGAGCACCTTCTCTTC-3' (257–276); 5'-GATCTTCACTTCCAGGTCGGC-3' (739–759). The PCR fragment, 257–759, was cloned into the pCRII vector (Invitrogen). Sequencing was carried out using a double-stranded DNA cycle sequencing protocol (BRL Life Technologies) using the following primers. The sense primers used were 5'-GGGGGAGCCTATGGGTGGGG-3' (496–516), 5'-CTGGACAAGGTGCGTGCTCTG-3' (706–726), 5'-ACAGCCACAGTGGACAATGCC-3' (2106–2126), 5'-AGCCTGAAGGAGGAGCTGGCC-3' (2857–2877), and 5'-GAGCTGCAGTCCCAGCTCAGC-3' (3583–3603). Antisense primers used were 5'-AGTGCTTGGGCAGGAGAGGGG-3' (4788–4808), 5'-CAGCTGCATGCAGTAGCGACC-3' (3737–3757), 5'-ACCATTCTCGGCATCCTTGC-3' (3366–3386), and 5'-GAAGTCATCCGCGGCCAGACG-3' (2154–2174). The conditions for reverse transcription, PCR, and sequencing were as described previously (Rugg et al. 1993b).

Screen for deletion mutation

Genomic DNA from 50 unrelated, unaffected individuals was PCR amplified using the primers: 5'-GGGGGAGCCTATGGGTGGGG-3' (496–516); 5'-GATCTTCACTTCCAGGTCGGC-3' (739–759) in the presence of [α -³²P]ATP (0.8 μ Ci/25 μ l reaction). The PCR conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and a final incubation of 72°C for 5 min. The product was analyzed on a 6% sequencing gel.

In situ hybridization probes and tissue sections

Keratin cDNAs (K1, K5, K10, and K14) were amplified by PCR from mRNA extracted from normal human primary keratinocyte cultures and cloned into pCR II vector (Invitrogen). Plasmids were linearized, and radioactive sense and antisense RNA probes were labeled with [α -³⁵S]CTP (800 Ci/mmol specific activity; Amersham) using SP6/T7 Transcription Kit (Boehringer). Incorporation of ³⁵S was checked, carrier tRNA was added, and probes were purified by phenol extraction and alcohol precipitation. Nonisotopic RNA probes were labeled with digoxigenin, using DIG RNA Labelling Kit (Boehringer), and purified by alcohol precipitation. RNase-free reagents, buffers, glass, and plastics were used up to the stage of washing after hybridization.

Skin biopsy specimens from the affected child and from an unrelated, unaffected individual were snap-frozen in Tissue-Tek O.C.T. Compound (Miles Inc). Sections of 7 μ m were placed on 3-aminopropyltriethoxysilane-coated slides (van Prooijen-Knegt et al. 1982). Sections were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, washed in PBS, digested with proteinase K for 15 min at 37°C, washed in dH₂O, fixed in 4% paraformaldehyde in PBS, washed in PBS, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature, washed in PBS, dehydrated in an ethanol series, and air-dried.

Radioactive in situ hybridization

Keratin mRNAs were assayed in situ using a modification of the protocol described by Harper et al. (1986). Sections from an unaffected individual were used as a control for probe and protocol specificity and sensitivity. ³⁵S-labeled cRNA probes (6×10⁵ cpm) for K5 and K14 supplemented with yeast tRNA were hybridized at 50°C overnight in 50% formamide, 2× SSC [0.3 M NaCl, 30 mM sodium citrate (pH 7.0), 1× Denhardt's solution, and 10 mM dithiothreitol. Sense probes were used as a negative control for background staining. The slides were washed in 2× SSC, 10 mM 2-mercaptoethanol, for 10 min at room temperature, digested with 20 μ g/ml of RNase A for 30 min at room temperature, washed 10 min at room temperature in 2× SSC, 10 mM 2-mercaptoethanol, twice for 30 min at 50°C in 0.5× SSC, 10 mM 2-mercaptoethanol, three times for 10 min at room temperature in 0.5× SSC, dehydrated in ethanol series with 0.3 M ammonium acetate, dried, immersed in Ilford K5 autoradiographic emulsion diluted 1:1 with 0.3 M ammonium acetate, and exposed for 7 days at 4°C. Slides were developed in Phenisol (Ilford) and fixed in Hypam (Ilford), counterstained in hematoxylin, and mounted in DPX.

Nonisotopic in situ hybridization

Digoxigenin-labeled probes for K1, K5, K10, and K14 were hybridized in 50% formamide, 2× SSC, 1× Denhardt's solution, and 1 mg/ml of tRNA at 50°C overnight. The K14 probe was hybridized to duplicate sections. Slides were washed in 2× SSC for 10 min at room temperature, digested with 20 μ g/ml of RNase A for 30 min at room temperature, washed 10 min at room temperature in 2× SSC, twice for 30 min at 50°C in 0.5× SSC, three times for 10 min at room temperature in 0.5× SSC, followed by DIG nucleic acid detection (Boehringer) according to the manufacturer's instructions. Alkaline phosphatase produced blue signal after overnight incubation with NBT/X-phosphate mixture. Slides were mounted in DPX without counterstaining of nuclei. Results were photographed with a 50× oil immersion lens.

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