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Inability of keratinocytes lacking their specific transglutaminase to form cross-linked envelopes: Absence of envelopes as a simple diagnostic test for lamellar ichthyosis

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ABSTRACT **Epidermal keratinocytes, late in their terminal differentiation, form cross-linked envelopes resistant to ionic detergent and reducing agent. Because the cross-linking process is catalyzed by the keratinocyte transglutaminase, the absence of active transglutaminase should result in failure of the keratinocyte to form a cross-linked envelope. Three keratinocyte strains bearing mutations in the keratinocyte transglutaminase were examined: two contained no detectable transglutaminase mRNA and none contained active enzyme. All three were unable to form cross-linked envelopes, either spontaneously in stratified cultures or upon induction with Ca^{2+}. Although stratum corneum of normal humans and scales from patients with different ichthyotic diseases contain cross-linked envelopes, those from patients with transglutaminase-negative lamellar ichthyosis do not. Therefore, the disease due to the absence of transglutaminase may be readily distinguished from other ichthyotic diseases by a simple test for cross-linked envelopes.**

The basal layer of the epidermis contains small cells, of which many are capable of multiplication, and the superficial layers contain larger cells that undergo terminal differentiation (1, 2). At the end of this process, the cells form cornified envelopes (3) consisting of cross-linked proteins (4). They then destroy their organelles and most soluble proteins and pass into the stratum corneum as corneocytes. The cross-linked envelope is not dissolved by boiling in the presence of ionic detergent and reducing agent and therefore is the most resistant component of the corneocyte (3).

The protein cross-linking that stabilizes the envelopes results from the action of the keratinocyte transglutaminase (TGase1) (5–8), which is anchored in the plasma membrane (9–12), and catalyzes the formation of isopeptide bonds between glutamine and lysine residues of neighboring polypeptides (13). The other two transglutaminases (TGases) found in the epidermis, tissue-type transglutaminase (TGase2) (14) and the epidermal transglutaminase (TGase3) (15–17) have no demonstrated role in envelope formation (18). All mammalian TGases are known to be activated by an increase in the cytosolic Ca^{2+} concentration.

Lamellar ichthyosis refers to a heterogeneous group of genetic skin disorders, some but not all of which are due to mutations in the TGase1 gene (19–26). Some of the common features in these diseases include a defective stratum corneum with reduced barrier function (27–29) and thinning of the marginal band of the corneocyte (30). By using cultured keratinocyte strains and epidermal scales from patients with ichthyoses, we show herein that, in the absence of TGase1, no cross-linked envelopes are formed. The diagnosis of TGase-negative lamellar ichthyosis can be readily made by simple inspection under the microscope.

MATERIALS AND METHODS

Cell Culture. Human epidermal keratinocytes were cultivated on lethally irradiated 3T3-J2 feeders in FAD medium containing supplements (31). For all the experiments, cells at lower than passage 5 were grown to confluence and harvested.

Northern and Western Blots. Total RNA was isolated by using Rneasy total RNA kit (Qiagen, Chatsworth, CA). Northern blot analysis was carried out according to ref. 32, with some modification. The TGase1 cDNA probe of 1.7 kb was released from the plasmid TG12 (obtained from R. Rice, University of California, Davis) (6) with *Eco*RI. As a control for loading variation, an oligonucleotide specific for 28S rRNA was used. For protein analysis, cells were harvested and lysed in 2× Laemmli sample buffer. Protein concentration was determined by the Bradford assay (Bio-Rad). Fifty microgram of cell extract was loaded onto a 7% PAGE gel. Western blot analysis was performed with an ECL detection kit (Amersham). The primary antibody to TGase1, a rabbit polyclonal antiserum against the C terminus (aliquot 13C) (33), was used at a 1:2,000 dilution.

TGase Assay. The assay was performed as described, with some modification (34–36). Keratinocyte cultures were harvested by scraping in 1 ml of buffer containing 20 mM Tris·HCl (pH 8.0), 2 mM EDTA, and leupeptin (Sigma; 0.5 μg/ml) and then sonicated. Fifty micrograms of the resulting extract was assayed in each reaction. The reaction volume of 50 μl contained 100 mM Tris·HCl (pH 8.0), 10 mM $CaCl_2$, 10 mM DTT, 2 μCi of ^{14}C-labeled glycine ethyl ester, 20% glycerol, and 200 μg of N,N-dimethylated casein as a substrate. For each keratinocyte strain, the background value was measured separately by adding 50 mM EDTA to the buffer before the protein extract. Reaction was carried out at 37°C for 1 h. Proteins were then precipitated with trichloroacetic acid and filtered, and radioactivity was measured. The enzyme activity was calculated by subtracting the value obtained in the presence of 50 mM EDTA from the reaction value and expressed in cpm per h per μg of extract protein. As a control, 7.2 milliunits of purified guinea pig liver TGase (Sigma) consistently incorporated 6×10^4 cpm.

Preparation of Cross-Linked Envelopes. For spontaneous envelope formation, cells were trypsinized, counted, and resuspended in 1 M Tris·HCl (pH 8.0) containing 2% 2-mercaptoethanol and 2% SDS. The suspension was heated to 100°C for 10 min and vortex-mixed for 10 min. Envelopes were counted by using a hemocytometer under a light microscope.

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Abbreviations: TGase, transglutaminase; TGase1, keratinocyte transglutaminase.

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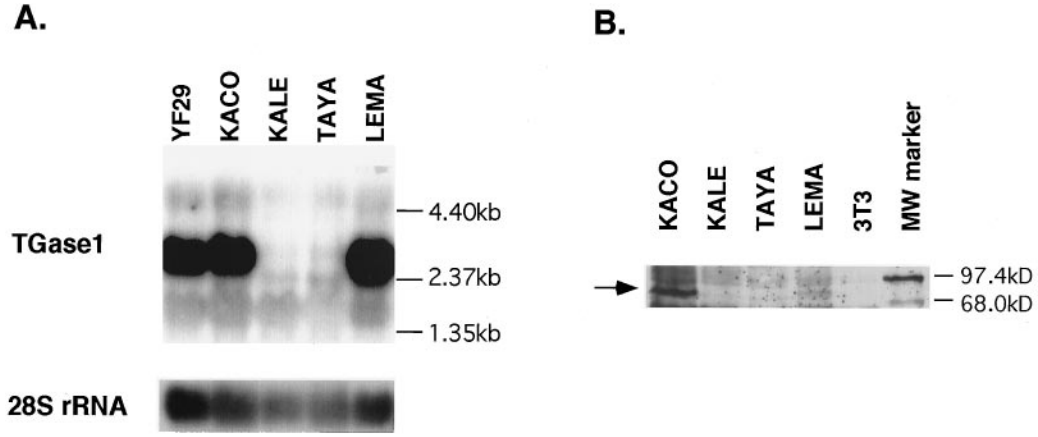

FIG. 1. Expression of TGase1 in wild-type strains (YF29 and KACO) and mutant strains (KALE, TAYA, and LEMA). (*A*) Northern blots hybridized with a TGase1-specific cDNA probe (*Upper*) and with an oligonucleotide probe specific for 28S rRNA (*Lower*). Positions of RNA molecular weight markers are shown on the right (*Upper*). The autoradiograph is overexposed to demonstrate clearly the absence of TGase1 mRNA in KALE and TAYA. (*B*) Western blot probed with a rabbit polyclonal antibody raised against a C-terminal peptide of TGase1. Arrow indicates the position of the 92-kDa TGase1. A protein extract prepared from 3T3-J2 cells was included as a negative control. Neither KALE nor TAYA produced an enzyme band but a faint band is present in extract of LEMA cells, which contain a missense mutation.

To accelerate formation of cornified envelopes, 500-μl aliquots of 0.5–1.0 $\times$ 10^6 keratinocytes in serum-free DMEM were incubated at 37°C with the calcium ionophore A23187 at 100 μg/ml for 0, 5, 15, 30, 60, and 120 min (5). At different times, 2% 2-mercaptoethanol and 2% SDS were added to an aliquot of cell suspension, and envelopes were counted after heating. To block the action of ionophore-induced TGase, 1 $\times$ 10^6 cells were incubated with the inhibitor RS48373, obtained from Seth Michelson (Syntex, Palo Alto, CA) (37) for 15 min, after which ionophore was added at a concentration of 50 μg/ml and incubated for 10 min. To examine scales from patients, 10 mg of sample was boiled for 10 min in the above-mentioned buffer, followed by vortex mixing.

RESULTS

Characterization of Keratinocyte Strains. To determine whether active TGase1 is essential for epidermal cells to form cornified envelopes, we examined three mutant strains of keratinocytes derived from patients with lamellar ichthyosis and two wild-type strains (KACO and YF29). Of two mutants, provided by Ariane Rochat (Ecole Normale Superieure, Paris), TAYA was known to be free of mRNA for TGase1 and LEMA was to possess the mRNA but not enzyme activity [patients A4 and 1, respectively, described in Petit *et al.* (26)]. The third mutant (KALE), provided by Irene Leigh (Queen Mary and Westfield College, London), was also from a patient diagnosed as typical lamellar ichthyosis, but the absence of the enzyme was not established.

To determine the presence of TGase1 mRNA in the cultured cells, total RNA was isolated from confluent cultures and subjected to Northern blot analysis using a DNA probe specific to TGase1 (Fig. 1*A*). A strong band of 2.9 kb was visible in the two wild-type strains (KACO and YF29). We confirmed that the mutant strain LEMA, known to harbor a missense mutation in the third exon of the gene, possessed the specific mRNA and that cells with a mutation in the promoter region (TAYA) lacked it (26). KALE also lacked TGase mRNA. All cell strains showed two minor RNA species migrating at the positions of ~4.5 and 1.9 kb in size, presumably due to cross-hybridization of the probe to tissue-type TGase (TGase2) (17, 18) and a TGase homologue, TGase-H, respectively (38).

The presence of the corresponding protein was tested by immunoblotting, using a rabbit polyclonal antibody raised against a C-terminal peptide of TGase1 (Fig. 1*B*) (33). A corresponding band with a molecular mass of 92 kDa was present in the wild-type strain KACO. The mutant strain LEMA, whose TGase1 had a single nucleotide substitution, produced a faint band of the expected size. The other two mutants lacking the TGase1 mRNA had no detectable protein.

The activity of TGase was measured by incorporation of ^{14}C-labeled glycine ethyl ester into a protein substrate, *N,N*-

Table 1. TGase activity in extracts of wild-type and mutant cell strains

	^{14}C incorporation, cpm per μg of protein per h		
Sample	Complete system (experimental)	Complete system + EDTA (background)	TGase-catalyzed reaction (experimental − background)
YF29	208	17.6	190
KACO	227	15.1	212
KALE	23.0	8.30	14.7
TAYA	26.2	22.1	4.12
LEMA	28.1	11.7	16.4

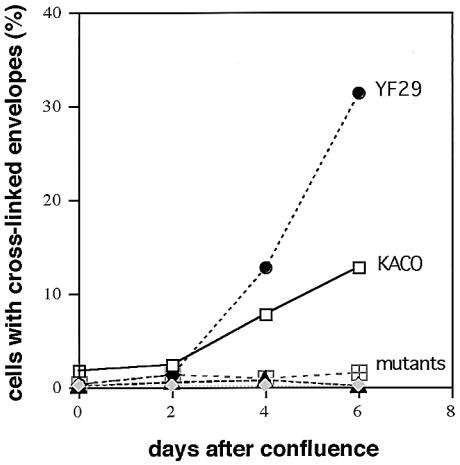

FIG. 2. Spontaneous envelope formation in wild-type and mutant strains. After heating to 100°C in the presence of SDS and 2-mercaptoethanol envelopes were counted. The numbers obtained are expressed as percentage of total number of cells and plotted as a function of time after confluence. Virtually no envelopes were produced by any of the three mutant strains.

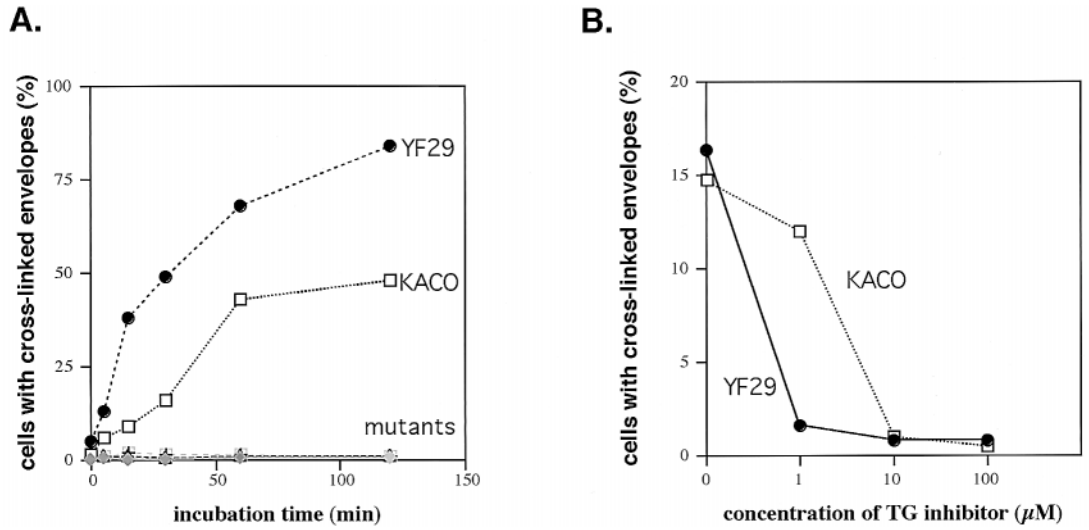

FIG. 3. Envelope formation after induction by calcium ionophore A23187. (*A*) Number of cross-linked cell envelopes as a function of incubation time in the presence of A23187 (100 μg/ml). There was virtually no envelope formation by any of three mutant strains. (*B*) The effect of TGase inhibitor RS48373 on envelope formation in wild-type cells treated with ionophore.

dimethyl casein. Incubation of the two substrates with extracts of wild-type keratinocytes (YF29) led to a linear increase in incorporation of labeled glycine ethyl ester into protein for a period of at least 1 h. Under the same condition, the three mutant strains, including LEMA, showed a mean TGase activity of 5.8% that of wild-type strains (Table 1). This activity may be attributed to tissue-type TGase2, the product of an independent gene (14, 39). A contribution of TGase3 is unlikely because TGase3 is not expressed in epidermal cultures (17).

Formation of Cross-Linked Envelopes. To relate TGase1 to the ability to form cornified envelopes, keratinocyte cultures were allowed to stratify and differentiate (3). At different times after reaching confluence, cells were trypsinized and counted. After addition of 2% SDS and 2% 2-mercaptoethanol and heating to 100°C for 10 min, cross-linked envelopes were counted and their number was plotted as a proportion of the number of cells (Fig. 2). In wild-type strains, the number of cross-linked envelopes increased with time and reached 10–30% of the total number of cells at 6 days after confluence. All three mutants formed either no envelopes at all or, in some cases, structures that could not be clearly distinguished from envelopes, but with a frequency less than 1% of the total number of cells.

In an independent and somewhat more sensitive assay (5), the formation of cornified envelopes was promoted by adding a calcium ionophore that activates TGase by raising the internal Ca^{2+} concentration (40). Confluent cells were trypsinized and 0.5–1 × 10^6 cells were incubated with ionophore A23187 at a concentration of 100 μg/ml for various periods (Fig. 3*A*). The wild-type strains produced envelopes in 50–80% of the cells, but the mutant strains showed no detectable envelopes at any time up to 2 h. The ability of wild-type keratinocytes to form envelopes depends on their TGase, because addition of the mechanism-based inhibitor halodihydroisoxazole RS48373 (37) prevented envelope formation in a concentration-dependent manner (Fig. 3*B*). Cultured cells lacking TGase1 therefore are unable to form envelopes by all tests applied.

Presence of Cross-Linked Envelopes in the Outer Layers of the Epidermis. Because all the mutant cell strains we analyzed were unable to form cornified envelopes, we examined the outer layers of cells of the epidermis for the presence of envelopes. Corneocytes were scraped from a healthy individual or patients with ichthyotic diseases and analyzed for detergent-resistant cross-linked envelopes. Corneocytes of normal epidermis possessed cross-linked envelopes that were insoluble in a solution containing 2% SDS and 2% 2-mercap-

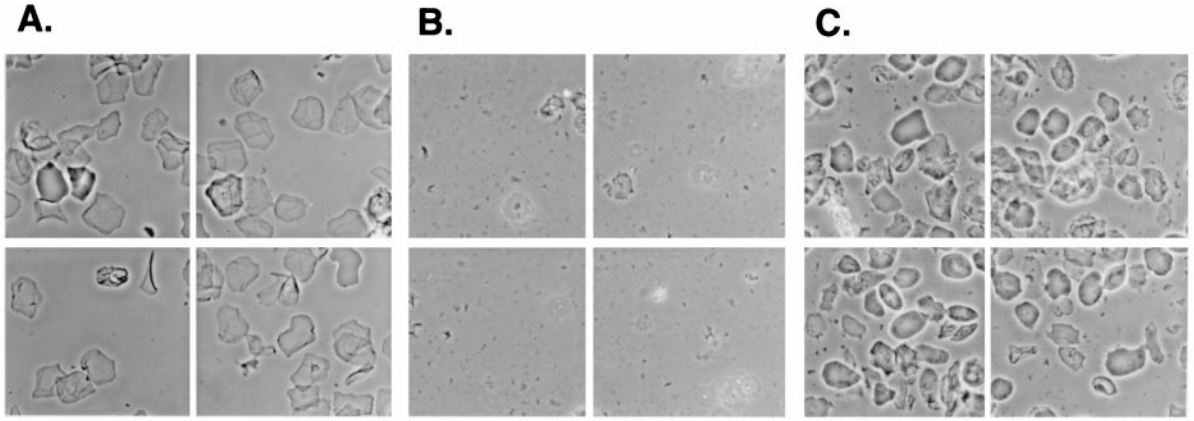

FIG. 4. Absence of cornified cell envelopes in scales from a patient with TGase-negative lamellar ichthyosis. (*A*) Cornified cell envelopes in corneocytes scraped from normal human. (*B*) Absence of envelopes in corneocytes from a patient with lamellar ichthyosis. (*C*) Presence of dense envelopes in corneocytes of a patient with Netherton syndrome. Assays for TGase in the insoluble fraction of these corneocytes gave values 10.6 times higher than those of *B*, whose small amount of enzyme activity we attribute to TGase2.

toethanol. The envelopes were frequently observed as polyhedra, presumably because they had formed in cells with close packing (Fig. 4*A*). Corneocytes from a patient with lamellar ichthyosis, provided by John Stanley (Univ. of Pennsylvania Health Service, Philadelphia), left no typical envelopes upon treatment with the detergent and reducing agent, although a few cell fragments could be detected (Fig. 4*B*). On the other hand, corneocytes (provided by Ehrhardt Proksch, Univ. of Kiel, Germany) from a patient with Netherton syndrome (20), another ichthyotic disease often confused with lamellar ichthyosis, showed well-defined envelopes that seemed denser than those of normal epidermis (Fig. 4*C*). Corneocytes from another patient diagnosed as having lamellar ichthyosis but found to possess TGase1 produced similar envelopes (data not shown).

It is therefore clear that different ichthyotic diseases that have been included in the group called lamellar ichthyosis may be distinguished as containing or lacking TGase1 by simple examination of lightly scraped or otherwise detached surface layers of the epidermis for the presence of cross-linked envelopes.

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