In Vitro and Rapid *In Situ* Transglutaminase Assays for Congenital Ichthyoses – A Comparative Study

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Autosomal recessive congenital ichthyoses are a heterogeneous group of disfiguring skin diseases. They are generally characterized by variable scaling and erythroderma, and patients are frequently collodion babies at birth. Autosomal recessive congenital ichthyoses are represented in 25 of our 50 families by a defective keratinocyte transglutaminase (TGK). Pathogenic classification is difficult to assess on clinical grounds for autosomal recessive congenital ichthyoses and impossible for collodion babies. Thus, we have established a rapid TGK assay *in situ* on frozen skin sections using incorporation of dansyl-cadaverin to assess transglutaminase (TG) activity in combination with immunohistochemistry for TGK protein. Results were compared with TG activity levels measured in cultured differentiating keratinocytes. Sixteen of 26 patients, including a collodion baby, had strongly diminished TG activity in the cell periphery of differentiating keratinocytes and membrane-bound TG activities *in vitro*, ranging from 2.2 to 281.3 pmol per h mg. Nine of 26 patients, including a collodion baby, showed strong TG activity in the cell periphery of differentiating keratinocytes *in situ* and membrane-bound TG activities *in vitro* ranged from 1519 to 10917 pmol per h mg. In one case, TG assay *in situ* was ambiguous; however, membranous TG activity *in vitro* was very low at 76.9 pmol/h × mg. Our results demonstrate an excellent correlation of TG assays *in vitro* and *in situ*. In addition, we present a novel test with prognostic value for the collodion baby phenotype. This assay allows rapid pathogenic classification of autosomal recessive congenital ichthyoses with only one caveat that in rare ambiguous cases it might be necessary for proper classification to assess membrane-bound TG activity *in vitro*. *Key words: keratinocyte/lamellar ichthyosis/transglutaminase. J Invest Dermatol 110:268–271, 1998*

Autosomal recessive congenital ichthyoses (ARCI, syn. ichthyosis congenita mitis, lamellar ichthyosis, nonbullous congenital ichthyosiform erythroderma, MIM # 242100, 242300, and 601277) are severe genodermatoses with generalized ichthyotic scaling as their most prominent feature (Traupe, 1989; Anton-Lamprecht, 1992). ARCI are clinically and genetically heterogeneous. The phenotype can range from large brownish plate-like scales with no erythroderma (i.e., lamellar ichthyosis) (Frost and Van Scott, 1966) to fine white scales with underlying erythroderma (i.e., nonbullous congenital ichthyosiform erythroderma) (Brocq, 1902). Moreover, patients may have palmar and plantar hyperkeratosis, scarring alopecia, ectropion, eclabium, and decreased sweating. Patients are often born encased in a shiny, thick parchmentlike membrane (collodion baby). ARCI are rare and have an estimated frequency of about 1:100,000–1:250,000.

Transglutaminases (TG) are a superfamily of enzymes that catalyze transamidation of glutamine residues, a reaction associated with a wide variety of physiologic processes such as blood clotting, cytoplasmic coagulation in apoptosis, keratinization, hair follicle formation, and fertilization (Greenberg *et al*, 1991; Polakowska and Goldsmith, 1991; Aeschlimann and Paulsson, 1994). Two members of this protein family, keratinocyte TG (TGK or TG1) and epidermal TG (TGE or TG3), mediate Nε-(γ-glutamyl)lysine cross-linkage (Thacher and Rice, 1985; Thacher, 1989) during formation of the cornified cell envelope (CE), a distinct and highly insoluble structure of 8–15 nm thickness that replaces the plasma membrane in terminally differentiating keratinocytes (Hohl and Roop, 1993; Reichert *et al*, 1993). This process involves the sequential cross-linking of CE precursor proteins such as involucrin, cystatinA, small proline rich proteins, elafin, and loricrin on the inner side of the plasma membrane (Hohl and Roop, 1993; Steinert and Marekov, 1995). Simultaneously, the plasma membrane is replaced by Ω-hydroxyacyl-sphingosine lipids covalently bound to the outer surface of the protein CE (Wertz *et al*, 1989; Steinert and Marekov, 1997). TGK is mostly expressed on the plasma membrane in upper spinous and granular cell layers of stratified squamous epithelia (Thacher and Rice, 1985; Thacher, 1989; Kim *et al*, 1995). Approximately 5–10% of TGK activity is found in the cytoplasmic fraction and may account for the final steps of CE cross-linkage (Thacher and Rice, 1985; Thacher, 1989). TGK activity requires a catalytic triad (Cys, His, Asp) arranged in the enzyme active site and is dependant on forming a complex with calcium ions (Greenberg *et al*, 1991; Aeschlimann and Paulsson, 1994; Pedersen *et al*, 1994). The human TGK gene contains 15 exons, is located on chromosome 14q11 (TGM1 locus), and has at least two sequence variants (Kim *et al*, 1992; Phillips *et al*, 1992)

Recently, faulty TGK expression (Hohl *et al*, 1993) caused by deleterious mutations in the TGK gene has been identified as a cause of ARCI (Huber *et al*, 1995a; Parmentier *et al*, 1995; Russell *et al*, 1995), providing compelling evidence for the importance of the CE for epidermal homeostasis and the barrier function of the skin.

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Abbreviations: ARCI, autosomal recessive congenital ichthyoses; CE, cornified cell envelope; TG, transglutaminase; TGE, epidermal transglutaminase, TG3; TGK, keratinocyte transglutaminase, TG1.

Table I. Correlation of TG assays *in vivo* and *in vitro*

Patient	Cytosol pmol/h mg	Membrane pmol/h mg	IF
LI-1.5	13.9 ± 7.6 (2)	44.6 ± 24.2 (2)	neg
LI-3.7	34.5 ± 2.5 (2)	76.9 ± 24.6 (2)	pos?
LI-3.8	26.3 ± 9.7 (2)	37.2 ± 22.8 (2)	neg
LI-5.3	208.8 ± 1.3 (2)	3837.2 ± 24.1 (2)	pos
LI-8.3	5.8 ± 1.2 (3)	13.9 ± 4.5 (3)	neg
LI-12.6	458.2 ± 1.3 (2)	1519.4 ± 225.6 (2)	pos
LI-20.1	166.0 ± 89.7 (2)	175.4 ± 62.1 (2)	neg
LI-20.3	2 ± 1.1 (2)	2.2 ± 0.4 (2)	neg
LI-20.4	5.6 ± 2.1 (2)	4 ± 2.6 (2)	neg
LI-22	4 ± 2.4 (2)	52.2 ± 11.0 (3)	neg
LI-23	37.9 ± 23.8 (3)	166.2 ± 51.7 (2)	neg
LI-24.3	2869.8 (1)	10917.7 ± 1797 (2)	pos
LI-24.4	2555.7 (1)	10719.7 ± 3626 (2)	pos
LI-27	684.3 (1)	5851.9 ± 1338 (2)	pos
LI-28	18.5 (1)	40.6 ± 2.5 (2)	neg
LI-30	8.5 ±4.6 (2)	31.9 ± 28.8 (2)	neg
LI-33	428.1 ± 306.4 (2)	7502.1 ± 4991 (2)	pos
LI-35	620.6 ± 399.5 (2)	6482.3 ± 2831 (2)	pos
LI-36	183.6 ± 4.2 (2)	1973.9 ± 457.6 (2)	pos
LI-38	9.1 ± 6.4 (2)	152.8 ± 118.5 (2)	neg
LI-39	42.9 (1)	180.4 (1)	neg
LI-40	632.5 (1)	6997.8 (1)	pos
83	16.5 ± 5.9 (2)	22.4 ± 19.0 (2)	neg
84	14.4 ± 9.4 (2)	10.1 ± 1.9 (2)	neg
87	26.5 ± 6.7 (2)	186.2 ± 78.7 (2)	neg
88	19.7 ± 1.0 (2)	281.2 ± 19.4 (2)	neg

Molecular and biochemical data, however, have clearly shown that about 50% of ARCI patients have normal TG activity (Huber *et al*, 1995b; Bale *et al*, 1996). Genetic heterogeneity was further supported by mapping studies identifying a second chromosomal locus on 2q33–35 and a third locus at a currently unknown location carrying disease-causing genes for ARCI (Parmentier *et al*, 1996). Because (i) pathogenic classification of ARCI is difficult on clinical grounds, (ii) prognosis for the collodion baby phenotype at birth is impossible, and (iii) information of genetic linkage analysis is mostly insufficient in small families, we attempted to establish reliable TG assays allowing proper and rapid classification.

MATERIALS AND METHODS

Patients Families with ARCI from Switzerland, Germany, France, Sweden, and the United States and normal volunteers from Switzerland were investigated (**Table I**). LI-5.3 and LI-8.3 were from those of collodion babies at the time of biopsy.

Cell culture Punch biopsies obtained from the trunk or proximal extremities of the probands were used to establish primary cultures of keratinocytes on lethally irradiated murine 3T3 fibroblasts (Huber *et al*, 1995a). Secondary cultures were grown in high calcium keratinocyte medium supplemented with 10% fetal calf serum. Five days post-confluency, total RNA and proteins were extracted for northern blot analysis and measuring TG activity as described below.

TG activity Cells were lysed by sonification in 20 mM sodium phosphate pH 7.2, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 10 mM dithiothreitol, 50 μg phenylmethylsulfonylfluoride per ml (cytosol fraction). After centrifugation at 25,000 × *g* at 4°C for 30 min, the pellet was re-extracted by sonification with the same buffer supplemented with 1% TritonX-100. Following incubation for 10 min at 37°C, the lysate was centrifuged as above and the supernatant (membrane fraction) collected for measuring the TG activity as described previously (Lichti *et al*, 1985; Huber *et al*, 1995a). Briefly, 5 μl of cell extract was added to 95 μl of the following freshly prepared solution: 10 μl of 0.5 M sodium borate pH 9, 5 μl of 10 mM EDTA pH 8, 5 μl of 100 mM $CaCl_2$, 20 μl of dimethylcasein (10 mg per ml), 2.5 μl of 10% Triton X-100, 0.5 μl of 1 M dithiothreitol, 2.8 μl of 100 μM putrescine, 1 μl of [1,4 (n)-^{3}H]putrescine dihydrochloride (1 mCi per ml, 10–30 Ci per mmol; NEN), and 48.2 μl H_2O. After incubation for 30 min at 28°C, 60 μl were applied to 3 MM cellulose filter paper (Whatman, Maidstone, U.K.), and washed sequentially in 10% trichloroacetic acid and 0.1% putrescine, 5% trichloroacetic acid and 0.05% putrescine, and 95% EtOH. We observed that the reaction is linear under these conditions; less than 5% of putrescine is utilized during the incubation. TG activity is expressed as pmol [^{3}H]putrescine incorporated into dimethylcaseine per h and mg protein. Results are shown as the mean of duplicate measurements performed on cell extracts from two cell passages.

Immunofluorescence Frozen sections were incubated in 100 mM Tris-Cl pH 8, 1% bovine serum albumin (BSA) for 30 min (to block nonspecific binding), and then in 100 mM Tris-Cl pH 8, 5 mM $CaCl_2$, 12 μM monodansylcadaverine for 1 h (to detect TG activity) (Aeschlimann *et al*, 1993). For a negative control in normal human skin, EDTA was added to a final concentration of 20 mM. After stopping the TG reaction in phosphate-buffered saline (PBS), 10 mM EDTA, sections were incubated with rabbit anti-dansyl antibody (1:100) (Aeschlimann *et al*, 1993) and monoclonal anti-TGK antibody B.C1 (1:1) (Thacher and Rice, 1985) in 12% BSA/PBS for 3 h. Sections were then incubated with biotinylated horse anti-mouse antibody (1:100) in 12% BSA/PBS and normal horse serum (1:100) for 30 min, followed by fluoroscein isothiocyanate labeled swine anti-rabbit antibody (1:40) and TexasRed streptavidin (1:400) in 12% BSA/PBS for 30 min. Reading and interpretation was performed by two independant observers.

RESULTS

***In vitro* TG assay** TG activities were measured in extracts obtained from well-differentiated cultured keratinocytes. The degree of differentiation was assessed by measuring involucrin mRNA levels in cells cultured under the same conditions as those used for determination of TG activities. Results from our 26 ARCI patients (see **Table I**) showed two obvious groups: one with low activities ranging from 2.2 to 281.3 pmol/h × mg with a mean ± SEM of 87.0 ± 20.6 pmol/h × mg (= TGK^- ARCI) and the other with high activities ranging from 1519 to 10917 pmol/h × mg with a mean ± SEM of 6200 ± 1122 pmol/h × mg (= TGK^+ ARCI). Various TGK mutations were identified in all 13 TGK^- ARCI patients analyzed by SSCP and DNA sequencing, but no mutations were observed in the three patients analyzed in the TGK^+ ARCI patients. A control group of seven normal healthy individuals showed values ranging from 3662 to 5030 pmol/h × mg with a mean ± SEM of 4179 ± 193 pmol/h × mg. Statistical analysis using the unpaired Student's t test showed that values from both the TGK^+ ARCI group and the healthy individuals were significantly different from the TGK^- ARCI group ($p < 0.0001$). No significant statistical difference was identified between the TGK^+ ARCI group and the healthy individuals.

In situ* TG assay** Analysis of normal human skin revealed the feasibility of the new TG assay *in situ*: after TGK activation by a high calcium buffer in the presence of dansylcadaverine, anti-dansyl antibodies decorated the cell periphery of keratinocytes in the middle stratum granulosum and more diffusely in the stratum corneum (**Fig 1*A) expressing TGK protein (**Fig 1*B***). It is interesting to note that two separate layers exhibited this activity. Addition of high amounts of EDTA to block TG activity concomitantly with the addition of the substrate leads to a drastic reduction of dansyl-cadaverin incorporation (**Fig 1*C***), whereas the protein staining pattern remained unchanged (**Fig 1*D***).

Much higher levels of cross-linkage of dansyl-cadaverin and expression of TGK were observed in epidermis from all TGK^+ ARCI cases *in vitro*. TG activity (**Fig 2*A***) and TGK protein (**Fig 2*B***) were predominantly located on cell peripheries in upper spinous and granular layers but homogeneously distributed in the stratum corneum. In contrast, in eight of 17 TGK^- ARCI individuals *in vitro*, TG activity was restricted to the stratum corneum (**Fig 2*C***) and no TGK protein was detected (**Fig 2*D***). Eight of 17 TGK^- ARCI cases were more difficult to interpret when slight staining for activity extended into the malpighian layers (**Fig 3*A***) but no TGK protein was detected (**Fig 3*B***). Interpretation was most difficult in one case (LI-3.7, see **Table I**) that exhibited a staining pattern of *in situ* TG activity and TGK protein (**Fig 3*C*, *D***) comparable with normal human skin (**Fig 1*A*, *B***).

DISCUSSION

Clinical classification of ARCI is controversial and reliable tools for clinical distinction of subtypes corresponding to pathogenic entities are actually lacking. Some authors have distinguished two subtypes, lamellar ichthyosis and nonbullous congenital ichthyosiform erythroderma (Williams and Elias, 1984; Traupe, 1989); however, both erythrodermic

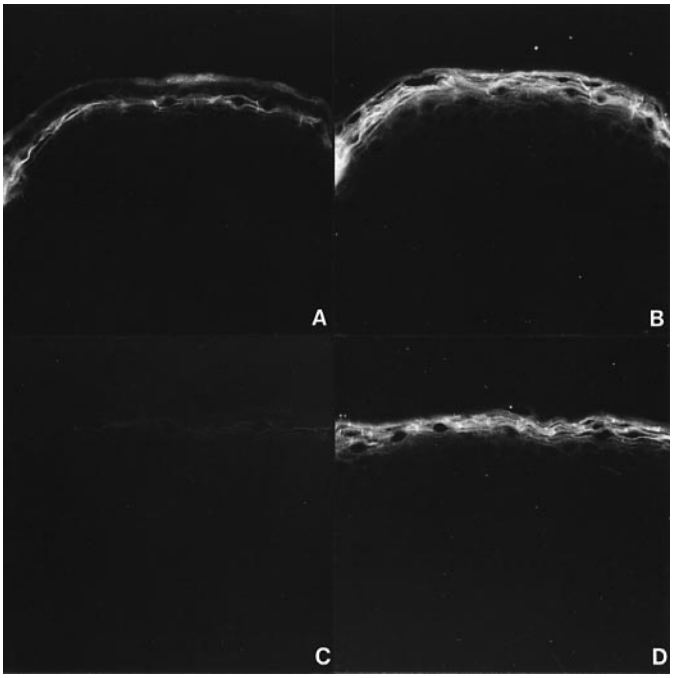

Figure 1. EDTA inhibits TG activity but not TGK protein expression. Analysis of normal human skin with anti-dansyl antibodies (*A*, *C*) and anti-TGK antibodies (*B*, *D*). EDTA added with the substrate leads to a drastic reduction of incorporation of dansyl-cadaverin (*C*).

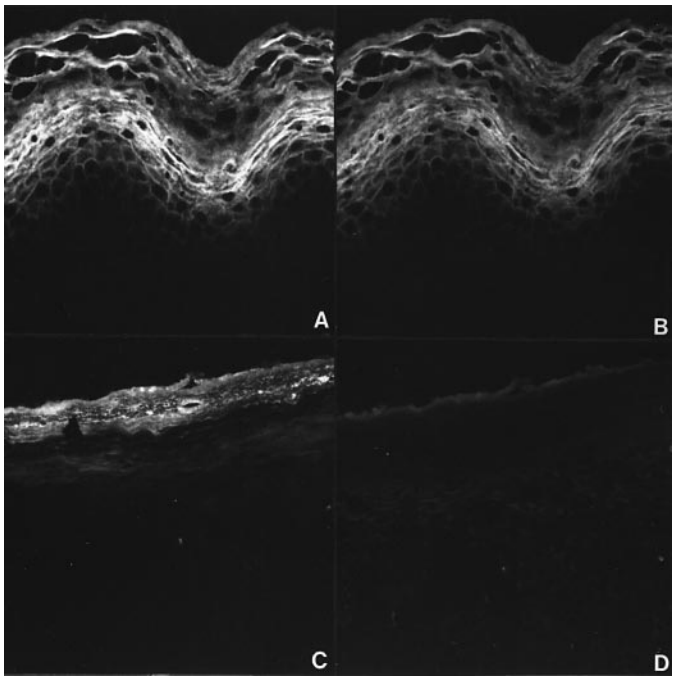

Figure 2. TGK deficiency is readily recognizable *in situ*. Analysis of skin from LI-24.4 (*A*, *B*) and LI-8.3 (*C*, *D*) with anti-dansyl antibodies (*A*, *C*) and anti-TGK antibodies (*B*, *D*). Incorporation of dansyl-cadaverin and expression of TGK is much higher in TGK^+ ARCI cases (*A*, *B*).

and nonerythrodermic patients with lamellar ichthyosis were found among TGK^- ARCI cases (Huber *et al*, 1995a). Moreover, recent molecular studies have indicated at least three disease-causing genes (Parmentier *et al*, 1996), and earlier ultrastructural studies suggested perhaps even up to four or five different types of ichthyosis congenita, indicating more complex heterogeneity (Anton-Lamprecht, 1992). In this study, we describe reliable and rapid biochemical tools for proper pathogenic classification of TGK^- ARCI cases, which account for 50% of all ARCI pedigrees analyzed in our series.

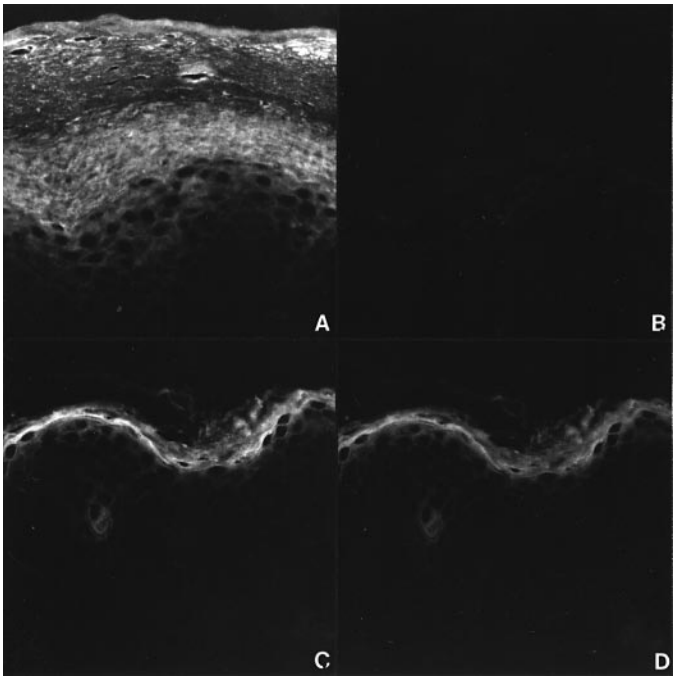

Figure 3. Analysis of uninvolved skin *in situ* may cause errors. Analysis of skin from LI-20.1 (*A*, *B*) and LI-3.7 (*C*, *D*) with anti-dansyl antibodies (*A*, *C*) and anti-TGK antibodies (*B*, *D*). LI-3.7 shows similar staining as normal human skin.

The TG assay *in vitro* (and also immunoblotting with the monoclonal antibody B.C1 to TGK, data not shown) can be considered as a gold standard for defining TGK^- ARCI cases biochemically. TGK^- and TGK^+ ARCI cases are clearly distinct with a more than 5-fold difference between the highest value in the TGK^- ARCI group and the lowest value in the TGK^+ ARCI group. The large variability of values measured within both groups may in part be due to the difficulty of standardizing keratinocyte differentiation in cell culture, because of different biopsy sites, different ages of patients, and distinct genetic backgrounds. The much wider spread of values in the TGK^- ARCI group (128-fold for TGK^- ARCI *versus* 7.2-fold for TGK^+ ARCI) nevertheless indicates clear differences in retained TG activities between different TGK mutants. This interpretation is supported by the pseudo-dominant pedigree LI-20. In this case, the affected mother LI-20.1 carried a different mutant allele and had a clearly higher TG activity than her two affected children that had identical TGK mutants and very similar TG activities. Furthermore, expression of different TGK mutants in identical TGK^- keratinocytes showed differences in their intrinsic activities, indicating that the remaining activity of each mutant is an inherent property of the position and nature of the mutation (Huber *et al*, 1997).

Our TG assay *in situ* showed an excellent correlation with the results from the cell cultures. Interpreting the activity assay required some experience due to the variable expression of the different TG throughout the epidermis. Weak TG activity and TGK protein expression was retained in the malpighian layers *in situ* in eight of 17 cases of the ARCI group TGK^- *in vitro* (**Fig 3*A*, *B***); however, this staining did not lead to false-positive results by two independent observers. The activity retained in the stratum corneum in all TGK^- ARCI cases (**Fig 2*C***) is probably caused by TGE activity (Park *et al*, 1988; Kim *et al*, 1993, 1994). As the staining of the stratum corneum in normal human skin was abolished by depletion of calcium (EDTA treatment; see **Fig 1*C***), nonspecific background staining is unlikely. Two reasons may contribute to the difficulties encountered with the biopsy from LI-3.7. This patient was previously reported to carry three point mutations and is one of the rare TGK^- ARCI patients expressing low levels of TGK protein detectable by immunoblotting with monoclonal antibody B.C1 (Huber *et al*, 1995a). Moreover, only tissue material from uninvolved skin was available for analysis. The drastic reduction

of TG activity in this patient only became apparent in cell cultures most likely due to the hyperproliferative conditions *in vitro*. The false-positive interpretation of TGK activity *in situ* could have been evaded by the use of involved acanthotic epidermis, as indicated by the correct interpretation of the biopsy obtained from the sister LI-3.8 carrying the same mutations. This seems to be a unique case/situation because we have never had similar difficulties in affected acanthotic epidermis.

In conclusion, we describe a reliable and rapid *in situ* TG assay that can be used as a diagnostic tool in dermatologic routine for ARCI and for collodion babies. We strongly recommend the use of involved skin for the assay *in situ*. If this is not possible, TG activity should be measured from differentiating keratinocyte cultures.

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