Short-Term Retinoic Acid Treatment Increases In Vivo, but Decreases In Vitro, Epidermal Transglutaminase-K Enzyme Activity and Immunoreactivity

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Epidermal transglutaminase-K is believed to catalyze the covalent linking of loricrin and involucrin to form cross-linked (CE) envelopes. In normal skin, transglutaminase-K is expressed as a band immediately below the stratum corneum, whereas in psoriasis and healing skin its expression is considerably expanded throughout the suprabasal layers. We have investigated whether the hyperproliferative state induced by short-term application of topical retinoic acid is similarly characterized by an increase in transglutaminase-K enzyme activity and immunoreactivity.

Retinoic acid (0.1% cream) or vehicle were applied to human skin and occluded for 4 d. Skin biopsies were obtained for measurement of transglutaminase-K and transglutaminase-C activity and immunoreactivity. For comparison, cultured normal human keratinocytes were incubated for 4 d in the presence of 1 μM retinoic acid and the subsequent transglutaminase-K activity and immunoreactivity measured. Transglutaminase-K activity was increased 2.8 times in retinoic acid compared to vehicle-treated skin ($p < 0.005$, $n = 12$) whereas there was no significant difference in transglutaminase-C activity. However, transglutaminase-K mRNA levels were not significantly different between retinoic acid– and vehicle-treated skin. In vehicle-treated skin, transglutaminase-K immunoreactivity was limited to a narrow, substratum corneal band, but was considerably expanded in a diffuse suprabasal pattern in retinoic acid–treated epidermis. In contrast, transglutaminase-K immunostaining was decreased and its enzymatic activity reduced sixfold in retinoic acid–treated keratinocytes ($p < 0.01$, $n = 4$).

These results demonstrate that retinoic acid treatment in vivo, in contrast to in vitro, leads to not only increased transglutaminase-K protein expression but also increased enzymatic activity in the absence of detectable increases in mRNA levels. These data, taken with the previously reported lack of in vivo modulation of the differentiation markers keratins 1 and 10 by retinoic acid, indicate that certain aspects of keratinocyte terminal differentiation that are altered in vitro by retinoic acid do not occur in vivo in human skin. *J Invest Dermatol 99:283–288, 1992*

The transglutaminases (Tgase) are calcium and sulfhydryl-dependent enzymes that modify proteins by forming covalent ϵ-(gamma-glutamyl) lysine cross-links [1]. To date, three types of Tgase have been described in epidermis including cytosoluble, pro-Tgase E [2,3], membrane-bound keratinocyte-specific transglutaminase (Tgase-K) [4–6], also termed type I or epidermal, and the cytosoluble tissue-type transglutaminase (Tgase C) or type II [7].

A prominent feature of keratinocyte terminal differentiation is the formation of a highly insoluble, cross-linked envelope (CE) immediately beneath the keratinocyte plasma membrane [8,9]. The production of keratinocyte CE is catalyzed by Tgase-K (and perhaps Tgase E), which cross-links the precursor proteins involucrin [10,11], loricrin [12], and keratolinin [13] via the formation of ϵ-(gamma-glutamyl) lysine isopeptide bonds. Using both immunohistochemical and monoclonal antibody techniques, Tgase-K and its substrate involucrin have been demonstrated in the upper layers of normal epidermis, situated immediately below the stratum corneum [4,14]. This finding is consistent with other markers of epidermal terminal differentiation such as keratins 1 and 10 [15].

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Abbreviations:
CE: cross-linked envelope
CRABP-II: cellular retinoic acid–binding protein-II
DMSO: dimethyl sulfoxide
EDTA: ethylenediaminetetraacetic acid
PBS: phosphate-buffered saline
RA: retinoic acid
Tgase: transglutaminase
Tgase-C: tissue type transglutaminase
Tgase-K: keratinocyte-specific transglutaminase

When added to keratinocytes in vitro, retinoic acid (RA) reduces Tgase-K activity and as a consequence may inhibit terminal differentiation and concomitant CE formation [16]. Paradoxically, RA is known to induce epidermal hyperproliferation and increased Tgase-K immunoreactivity when applied topically to normal human skin for 4 months in the treatment of photodamage [17] or for 4 d under occlusion [18]. Although epidermal Tgase-K immunoreactivity is increased after application of RA, it is not known whether this represents an increase in functional, cross-linking activity of the enzyme.

Using immunohistochemical and biochemical techniques, we have investigated the expression of Tgase-K enzyme activity, immunoreactivity, and mRNA in human skin treated with topical RA under occlusion for 4 d. The effects of RA on Tgase-K in vivo have been compared to those obtained in vitro with cultured human keratinocytes.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals used were obtained from the Sigma Chemical Company, St. Louis, MO, and were of at least reagent grade.

Tissue Samples Five hundred milligrams of 0.1% RA cream (Retin-A, Ortho Pharmaceutical Corporation, Raritan, NJ) and its vehicle were applied to two areas of the buttock skin of normal volunteers and the two sites occluded with Saran Wrap for 4 d as previously described [19]. All subsequent biopsies were obtained under local, 1% plain lidocaine, anesthesia. Keratome biopsies were obtained using an electric keratome set to a depth of 0.2 mm. Biopsies were immediately snap-frozen in liquid nitrogen and stored at −70°C until use. Four-millimeter punch biopsies taken for involucrin staining were placed in 10% formalin and processed to paraffin, whereas biopsies for Tgase-K staining were snap-frozen in liquid nitrogen and stored at −70°C until use. All subjects provided written, informed consent and all procedures were performed under approval of the University of Michigan Medical Center Institutional Review Board.

Cell Cultures Single-cell suspensions of normal, human keratinocytes were prepared from keratome biopsies of normal epidermis by trypsinization using 0.25% trypsin and 0.1% ethylenediaminetetraacetic acid (EDTA) for 30 min at 30°C. Small, round viable cells were seeded onto, and grown in, 10 cm diameter plastic dishes (Lux Flow Labs Inc., New York, NY) in a serum-free keratinocyte growth medium containing low calcium (modified MCDB 153). Cells were maintained in a humidified incubator with 5% CO_2/95% air at 37°C and used between passages 2–4. Cells were treated at 50% confluence with either RA [1 μM in dimethyl sulfoxide (DMSO)] or DMSO alone at a final concentration of 0.1% for 4 d. Previous experiments had determined that 0.1% DMSO had no effect on keratinocyte growth or morphology.

Measurement of Tgase Activity Tgase activity was measured using a modification of the technique of Yuspa et al [20]. In brief, frozen keratome biopsies were ground with a mortar and pestle and the powder suspended in homogenizing buffer (10 mM Tris, 10 mM dithiothreitol, 0.5 mM EDTA pH 7.4) and homogenized in a glass homogenizer. Resultant samples were centrifuged at 150,000 × *g* for 40 min at 4°C. The supernatant was decanted and kept on ice. The pellet was resuspended in homogenizing buffer containing 1% Triton X-100, incubated for 5 min at 37°C in order to solubilize the Tgase-K, and centrifuged as above to obtain a solubilized fraction. The efficiency of extraction by this procedure was found to be 70% ± 3% (n = 6).

Twenty microliters of the sample (decanted supernatant for Tgase-C or solubilized fraction for Tgase-K) were added to 20 μl of 500 mM sodium borate (pH 9.5), 10 μl of 10 mM EDTA (pH 7.5), 20 μl of casein (20 mg/ml), and 10 μl of [^{3}H] putrescine, and made up to 200 μl with 110 μl of distilled water. The subsequent reaction was conducted at 30°C for 30 min and terminated by the addition of 1 ml 10% TCA (4°C) containing 1% putrescine. Precipitated protein was collected on Whatman GF/C glass fiber filters. The filters were rinsed with 10 ml 10% TCA containing 1% putrescine dissolved in 0.5 ml protosol and counted in 10 mls of scintillation fluid.

Protein was measured by the method of Bradford [21] using gamma-globulin as standard, and DNA content was measured by the method of Burton [22].

Immunohistochemical Staining of Transglutaminase and Involucrin: Immunoperoxidase Five-micrometer cryostat sections were placed on welled, glass slides and air dried. After fixing in cold acetone, the sections were incubated for 45 min with a mouse monoclonal antibody to human Tgase-K [23] (BC1, Dr. S. M. Thacher, Texas A & M University) in a humidified chamber. After washing in phosphate-buffered saline (PBS), the sections were stained using a well-characterized avidin-biotin, immunoperoxidase technique (Vectastain ABC, Vector Laboratories, Burlingame, CA), with 3-amino-9-ethyl carbazole as the chromogen. The sections were counterstained with 1% hematoxylin.

Human keratinocytes grown in Lab-Tek chamber slides (Miles Inc., Elkhart, IN) were stained for Tgase-K in the same manner as for tissue sections following decanting of culture medium from the wells.

Five-micrometer sections cut from formalin-fixed, paraffin-embedded specimens were stained using an antibody to involucrin (Involucrin Immuno Kit, Biomedical Technologies, Stoughton, MA).

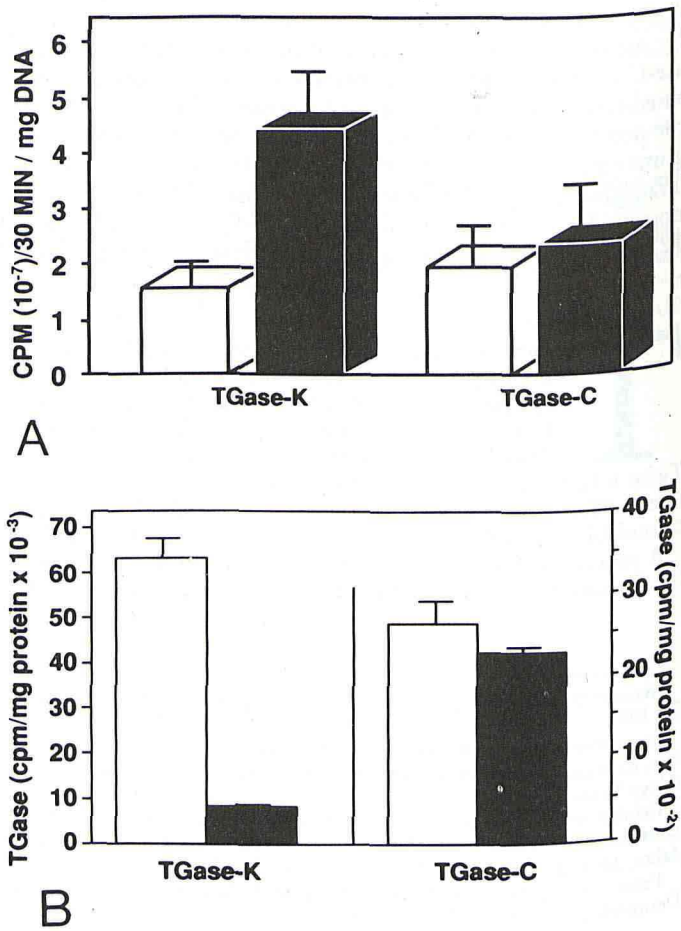
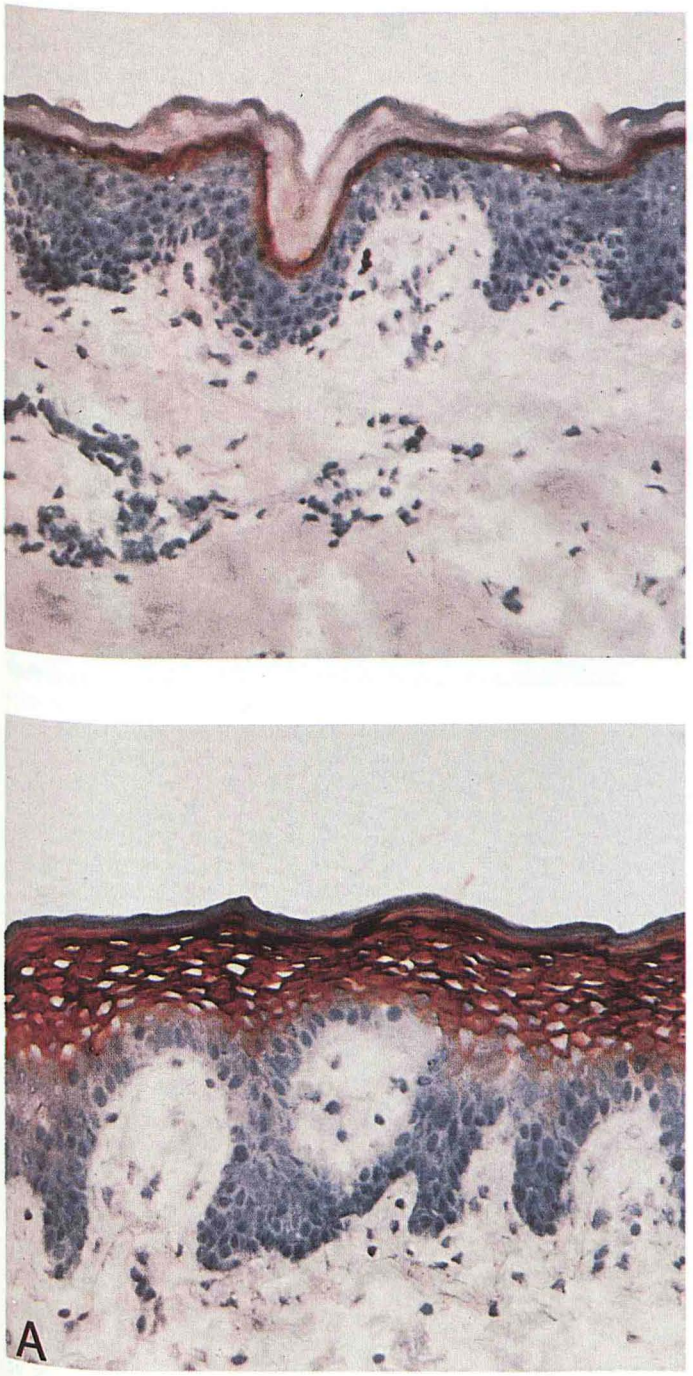
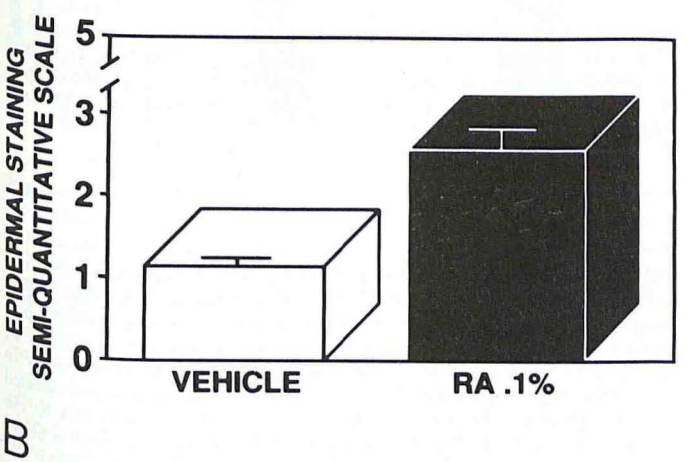


Figure 1. Effects of 4-d RA-treatment on TGase-K and TGase-C activities in vivo and in vitro. *A*, measurement of TGase-K and TGase-C activities in keratome biopsies from RA- and vehicle-treated skin. *Bars,* means ± SEM; n = 12, $p < 0.005$. *B*, measurement of Tgase-K and TGase-C activities in cultured adult human keratinocytes treated with RA (1 μM) for 4 d. *Bars,* means ± SEM. n = 4, $p < 0.001$. *Solid bars,* RA; *open bars,* vehicle.





A

B

The degree of epidermal staining with Tgase-K was assessed by light microscopy using a semiquantitative 1–5 scale where 1 = 0–20% of epidermis positive for Tgase-K; 2 = 21–40%; 3 = 41–60%; 4 = 61–80%; and 5 = 81–100% in five high power fields per individual. Involucrin staining was assessed in the same way.

Dansylcadaverine Fluorescence Five-micrometer cryostat sections of biopsies from RA and vehicle-treated skin (n = 3) were examined for fluorescence using previously described dansylcadaverine methodology, which identifies in situ enzyme activity [14,24]. Treated sections were examined using a Nikon microscope equipped with an appropriate excitation filter (DM 400) and emission filter (BA 460).

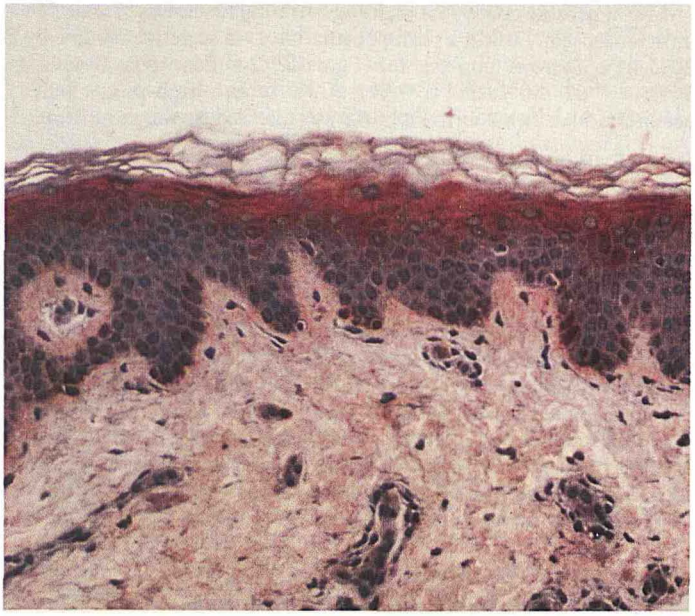
Indirect Immunofluorescence Five-micrometer biopsy sections were incubated overnight with either affinity-purified rabbit antisera directed against human Tgase-C (Dr. V. Thomazy, University Medical School, Debrecen, Hungary) or BC1. Slides were washed twice in PBS, once in distilled water, and incubated with secondary antibody for 30 min at room temperature. For the Tgase-C antibody, a fluorescein-conjugated swine–anti-rabbit antibody (Dako Corp., Carpinteria, CA) was used. For BC1 (anti Tgase-K), a biotinylated goat–anti-mouse secondary antibody was used (Vector Laboratories, Burlingame, CA). After washing in PBS and distilled water, the slides were incubated for 30 min with Texas Red–conjugated strepavidin (Gibco BRL). Following incubation and washing, the slides were mounted in polyvinyl alcohol. Stained cells were observed with a Nikon Labophot fluorescence microscope equipped with a BZE filter (fluorescein) or G filter (Texas Red).

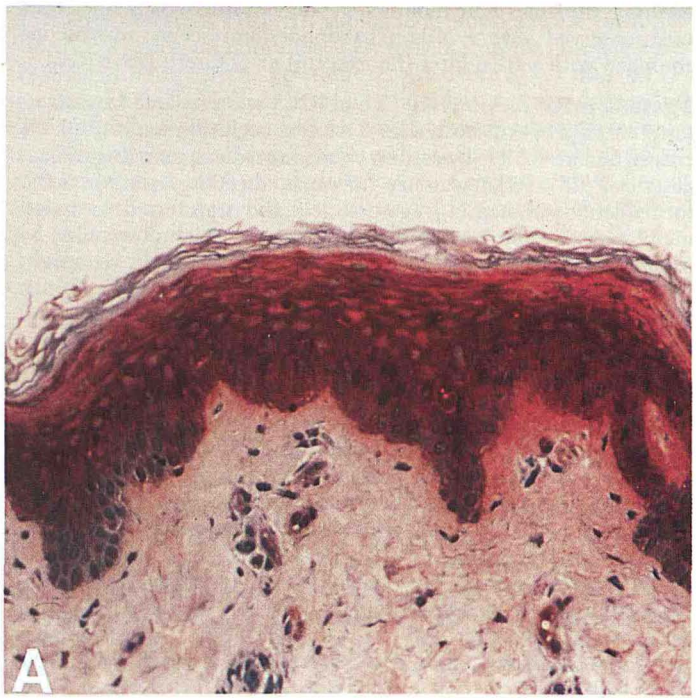
Tgase-K mRNA Analysis Total RNA was prepared from keratome biopsies of skin treated for 4 d under occlusion with RA (0.1% cream) and its vehicle by cesium chloride gradient centrifugation as described [25]. RNA was size fractionated (40 μg/lane) on 1.2% formaldehyde-containing agarose gels and transferred to nylon membrane (Zetaprobe, Bio Rad Laboratories, Richmond, CA) by passive capillary action. Membranes were hybridized overnight with randomly labeled cDNA probes for human Tgase-K, human cellular retinoic acid–binding protein-II (CRABP-II) [26], and human cyclophilin [27] at 42°C in STARK's buffer, salmon sperm DNA (10 mg/ml), and 10% dextran sulfate. Membranes were washed sequentially in 2 × SSC and 0.1 × SSC for 30 min at 55°C. Quantitation of mRNA bands was performed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Data are expressed as counts hybridized to the transcripts for Tgase-K or CRABP-II divided by counts hybridized to the cyclophilin transcript. The band intensity of cyclophilin was used as an internal control for differences in sample loading.

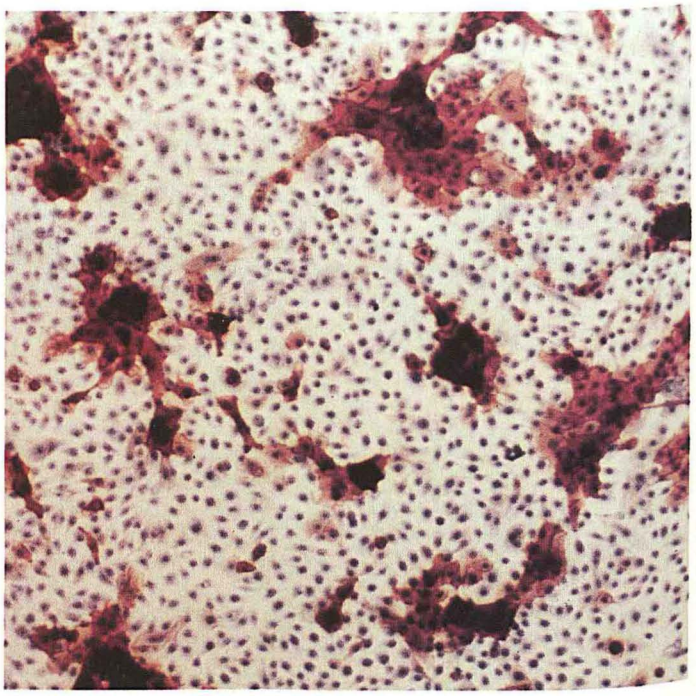
The human Tgase-K cDNA probe was cloned from a λgt11 keratinocyte library (Clontech, Palo Alto, CA), and contained 1.2 kb of the Tgase-K transcript, which had 100% homology to that described by Polakowska [28]. A full description of the cloning of the Tgase-K cDNA will appear elsewhere.

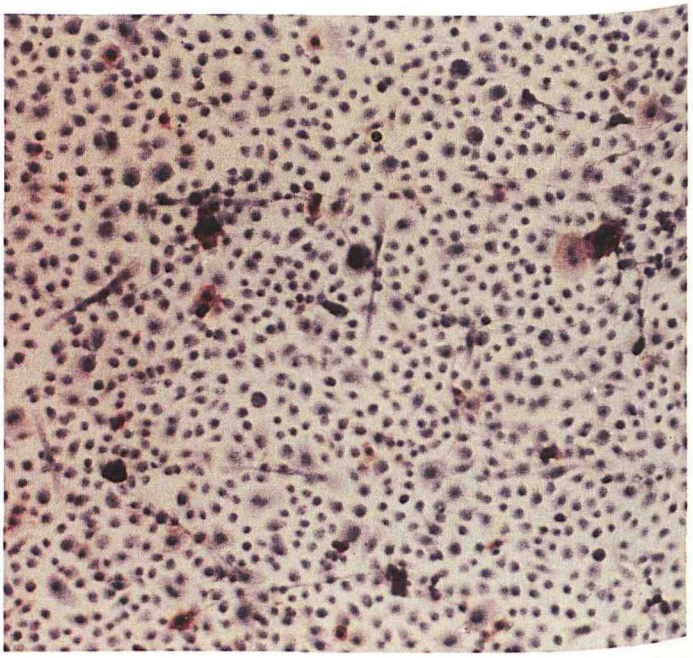
Statistical Analysis RA and vehicle effects on in vivo Tgase-K and involucrin immunoreactivity were compared using the Wilcoxon signed rank test. All other comparisons were made with a paired student t test.

Figure 2. Four-day RA treatment of normal skin causes increased TGase-K immunoreactivity. *A*, sections from normal human skin treated with vehicle *(top panel)* and 0.1% RA cream *(bottom panel)* for 4 d were stained with monoclonal antibody BC1 to Tgase-K. Visualization was by the immunoperoxidase method (× 230). *B*, semiquantitative assessment of Tgase-K immunoreactivity. The extent of TGase-K epidermal staining was assessed in five high-power fields from individuals treated with retinoic acid (0.1% cream) and its vehicle for 4 d. A 1–5 semiquantitative scale was used with 5 being maximum. *Bars*, means ± SEM. n = 18; vehicle versus RA $p < 0.001$.









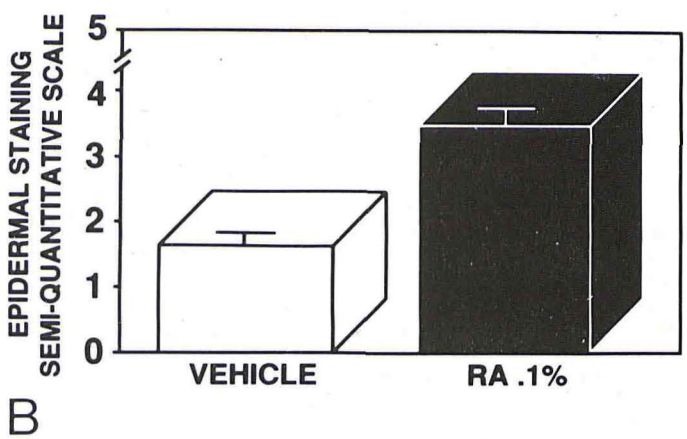


Figure 4. RA reduces TGase-K staining in cultured human keratinocytes. Keratinocytes were treated with 0.1% DMSO *(top panel)* or 1 μM RA in DMSO *(bottom panel)* for 4 d. Cells were stained for TGase-K with BC1 monoclonal antibody as described in *Materials and Methods.* (Magnification × 230.)

Figure 3. Four-day RA treatment of normal skin increases involucrin immunoreactivity. *A,* sections from human skin treated with RA vehicle *(top panel)* and 0.1% RA cream *(bottom panel)* were stained for involucrin. Visualization was by the immunoperoxidase method (magnification × 230). *B,* semiquantitative assessment of epidermal involucrin immunoreactivity in RA- and vehicle-treated skin. The extent of involucrin staining was assessed in five high power fields from individuals treated for 4 d with RA (0.1% cream) and its vehicle. A semiquantitative 1–5 scale was used, with 5 being maximum. *Bars,* means ± SEM. n = 5; vehicle versus RA, $p = 0.005$.

RESULTS

Effect of Retinoic Acid on Tgase-K and Tgase-C Enzyme Activities In Vivo and In Vitro Four-day application of 0.1% RA increased cutaneous Tgase-K activity 2.8 times as compared to 4-d treatment with vehicle alone ($n = 12$, $p < 0.005$). There was no significant difference in the activity of Tgase-C in human skin following treatment with 0.1% RA or vehicle cream ($n = 12$, $p = 0.74$), (Fig 1*A*).

Conversely, 4-d incubation of cultured keratinocytes with 1 μM RA produced a sixfold reduction in Tgase-K activity compared to control ($n = 4$, $p < 0.001$). There was no significant difference in Tgase-C activity between RA-treated or control keratinocytes (Fig 1*B*).

Effect of Retinoic Acid on Tgase-K and Tgase-C Immunoreactivities In Vivo and In Vitro In vehicle-treated skin, Tgase-K immunoreactivity was limited to a thin, compact, immediately subcorneal band (Fig 2*A*), whereas in 0.1% RA-treated skin, Tgase-K immunoreactivity was considerably expanded throughout the epidermis in a "chicken-wire" pattern (see Fig 2*A*). Using dansylcadaverine, we observed expanded epidermal staining in RA but not in vehicle-treated skin (data not shown), a pattern similar to that for the Tgase-K protein expression. Dansylcadaverine reactivity indicates potential in situ enzyme activity of Tgase-K [14], implying that the observed increase in protein expression is associated with an increased content in vivo of active enzyme. Using a semiquantitative scale from 1 to 5 there was a highly significant difference ($p < 0.001$) between RA- and vehicle-treated skin (Fig 2*B*). Involucrin reactivity coincided exactly with the staining pattern seen for Tgase-K in vehicle- and RA-treated skin (Fig 3*A*), being significantly increased in RA, compared to vehicle-treated skin ($p = 0.005$, Fig 3*B*). In no skin sample was Tgase-C immunoreactivity observed.

In contrast, treatment of cultured adult human keratinocytes for 4 d with RA (1 μM) resulted in greatly diminished Tgase-K immunostaining (Fig 4). This is consistent with the observed decrease in Tgase-K activity in keratinocytes following 4-d RA treatment (Fig 1*B*).

Tgase-K mRNA Levels Previous reports have demonstrated that treatment of cultured keratinocytes with RA is associated with a reduction in Tgase-K mRNA levels [29]. This finding is consistent with reduced Tgase-K activity in RA-treated keratinocytes and is evidence suggesting that Tgase-K expression is, at least in part, transcriptionally regulated [28,30]. Thus, one would expect that increased Tgase-K activity observed in RA-treated skin in vivo would be associated with elevated Tgase-K mRNA. Northern analysis of total RNA prepared from paired biopsies of RA- and vehicle-treated skin, from nine individuals, revealed a small (78%) elevation in Tgase-K mRNA in RA- compared to vehicle-treated skin (Fig 5*A*). This difference, however, was not statistically significant ($p = 0.14$). To serve as a positive control, the same RNA blots used for measurement of Tgase-K mRNA were reprobed for CRABP-II mRNA (Fig 5*B*). It has recently been shown that the mRNA levels of CRABP-II are significantly elevated in skin following 4-d RA treatment [26]. As expected, CRABP-II mRNA was found to be significantly elevated (threefold, $p < 0.002$) after RA treatment, indicating that failure to observe increased Tgase-K mRNA in RA-treated skin was not likely due to methodologic considerations.

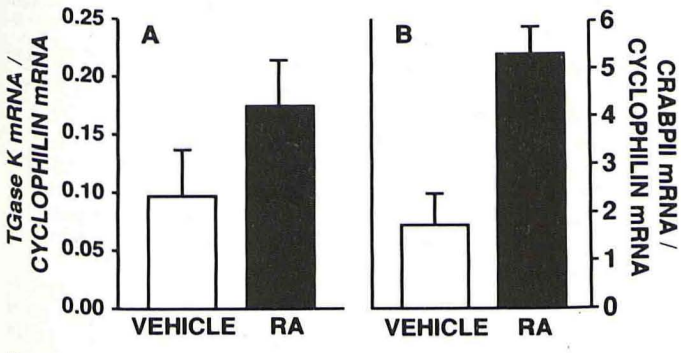


Figure 5. Tgase-K and CRABP-II mRNA levels in epidermal biopsies from human skin treated with RA and its vehicle. *A*, total RNA was prepared from keratome biopsies from 9 subjects treated with RA (0.1% cream) and its vehicle for 4 d under occlusion. Tgase-K mRNA was detected by Northern analysis (40 μg/lane) as described in *Materials and Methods*. The amount of [^{32}P]Tgase-K cDNA probe that hybridized to the 2.9-kb Tgase-K transcript was determined using a phosphorimager. The same RNA blot was hybridized to a cDNA probe for cyclophilin, and the amount of radioactivity hybridized to the Tgase-K mRNA band was normalized to that for cyclophilin. Vehicle vs RA, $p = 0.14$. *B*, the RNA blot was hybridized to the cDNA probe for CRABP-II, and the data were analyzed and expressed as done in *A*. Vehicle versus RA, $p = 0.001$.

DISCUSSION

This study demonstrates that short-term, occlusive application of RA to normal skin in vivo results in increased immunoreactivity and enzymatic activity of Tgase-K in conjunction with increased immunoreactivity of one of its substrates, involucrin. In contrast, RA reduces Tgase-K activity and immunoreactivity in cultured human keratinocytes. Rosenthal et al have also observed increased Tgase-K immunoreactivity in human skin treated with RA for 4 d [18].

Increased Tgase-K immunoreactivity has been reported in skin treated with RA for 16 weeks, although whether this represented an increase in functional, cross-linking activity of the enzume was not determined [17]. This study has shown that Tgase-K immunoreactivity is elevated within 4 d of RA treatment and that this correlates with increased enzymatic activity, which suggests that the increased Tgase-K immunoreactivity observed in chronically RA-treated skin would also represent increased enzymatic activity. Other epidermal hyperproliferative states, such as psoriasis [31,32] and wound healing [33,34], are also characterized by a suprabasal, precocious expression of Tgase-K immunoreactivity similar to our observations in RA-treated skin. Indeed, in psoriasis this increase in immunoreactivity is concomitant with a significant elevation of enzyme activity [35].

We have confirmed previous in vitro observations that RA reduces Tgase-K activity in cultured keratinocytes. It has recently been demonstrated that after a 4-d application of 0.1% RA under occlusion, levels of RA in keratome biopsies are in the 1–2-μM range [36]. Thus, the concentration of RA (1 μM) used in in vitro keratinocyte culture work is pertinent to the situation observed in vivo. It is currently unknown why RA exerts a paradoxic effect on Tgase-K and other markers of terminal differentiation in vitro [16,37] compared to that seen in vivo. The differential effect cannot be explained by RA-induced toxicity or inhibition of keratinocyte growth in vitro as we did not observe cell death nor loss of viability. One explanation may be the presence of cytokines, growth factors, and other growth-modifying agents within the epidermis and especially the dermis in vivo. These agents could interact with RA, either directly or indirectly, via modulation of RA receptors and binding proteins, thereby modifying cellular responses to RA. In addition, the role of calcium in vivo is undetermined and fluxes in the intracellular and extracellular concentrations of this cation may also act to modulate cellular responses to RA. Lichti et al have reported that RA treatment of mouse keratinocytes in vitro will induce Tgase-C [5]; by contrast this was not the case in humans either in vitro or in vivo. The reason for this difference is unknown.

Although the level of Tgase-K mRNA tended to be greater in RA- versus vehicle-treated skin, the difference between these two treatment groups failed to reach statistical significance. This was not due simply to variation in Tgase-K mRNA among the samples, because CRABP-II mRNA levels in the same samples could be shown, as expected [26], to be elevated significantly. It is possible that Tgase-K mRNA may have been transiently induced prior to the fourth day following RA treatment, when biopsies were taken for analysis. Alternatively, induction of Tgase-K activity in RA-treated

skin may involve predominently post-transcriptional or post-translational mechanisms that have been described in the regulation of keratin expression [38].

An increase in involucrin and Tgase-K in RA-treated skin would signify a maintenance of the cutaneous barrier function in long-term treatment, an observation that would not have been predicted from purely in vitro studies with cultured human keratinocytes. The 4-d occluded retinoid bioassay [19] may prove to be a predictive model for the alterations in epidermal proliferation and differentiation observed in chronic treatment of photoaging with RA cream. This model system also allows the implementation of functional biochemical assays, which require larger biopsy specimens than can be obtained in RA-treated facial or forearm photodamaged skin.

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