A Novel Approach for the Detection of Proteolytically Activated Transglutaminase 1 in Epidermis Using Cleavage Site-Directed Antibodies

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It has been suggested that transglutaminase 1 is proteolytically activated upon the terminal differentiation of the keratinocyte, but the mechanisms are not well understood. We have established two mouse hybridoma cell lines producing monoclonal antibodies that specifically detect proteolytically cleaved transglutaminase 1. One detects the amino-terminus of the fragment produced by cleavage between Arginine 93 and Glycine 94, and the other detects the amino-terminus of the fragment produced by cleavage between Arginine 573 and Glycine 574. Using these two antibodies, immunohistochemical analyses of the epidermis revealed that the cleavages of the transglutaminase 1 protein occur early in the terminal differentiation of keratinocytes in the basal layer of the epidermis, that the cleavage between Arginine 573 and Glycine 574 (producing the 574G fragment) precedes the cleavage between Arginine 93 and Glycine 94 (producing the 94G fragment), that the 94G fragment is localized to the plasma membrane of keratinocytes and has cross-linking activity, whereas the 574G fragment is dispersed in the cytosol and does not have detectable levels of activity on *in situ* transglutaminase assay, and that 1-α-25-dihydroxycholecalciferol or all-*trans* retinoic acid treatment and ultraviolet B exposure disturb the localization of the transglutaminase 1 fragments with changes in the morphology of differentiating keratinocytes. All these results demonstrate that the antibodies generated in this work are useful to dissect the mechanism by which transglutaminase 1 is activated, and would provide us with novel insights into the biogenesis of the epidermis. ***Key words: antibodies/cell differentiation/epidermis/keratinocyte/transglutaminase 1. J Invest Dermatol 121:457–464, 2003***

Transglutaminases (TGase; EC 2.3.2.13) are calcium-dependent enzymes that catalyze the formation of covalent bonds between the glutamyl residues and lysyl residues of either proteins or polyamines (Greenberg *et al*, 1991). These reactions are observed in cells and tissues from bacteria to vertebrates, and result in stable bonds, resistant to chemical, enzymatic, and physical degradation (Tokunaga *et al*, 1993; Cariello *et al*, 1997; Kobayashi *et al*, 1998; Makarova *et al*, 1999).

Among several isotypes of TGase in mammals, TGase1 and TGase3 are thought to contribute to maintain the toughened skin epidermis through cross-linking activity (Greenberg *et al*, 1991). During the terminal differentiation of keratinocytes, some structural proteins are cross-linked by TGase1 and/or TGase3 to form a cornified envelope (CE) that acts as an important barrier against water loss in the skin epidermis (Yaffe *et al*, 1992; Candi *et al*, 1995). Recently, it was reported (Nemes *et al*, 1999b) that TGase1 has the ability to attach ω-hydroxyceramide, an important lipid in the formation of CE, to involucrin, one of the structural proteins involved in CE formation (Yaffe *et al*, 1992). The resultant protein–lipid complexes probably improve the function of the CE in preventing water loss. Loss of TGase1 activity in mice results in early neonatal death at least partly due to the failure of CE formation (Matsuki *et al*, 1998), suggesting that TGase3 activity cannot compensate for the function of TGase1. Therefore, TGase1 is regarded as one of the most important enzymes for the construction of the CE (Thacher and Rice, 1985; Kuramoto *et al*, 2002).

TGase1 is synthesized as an 817-residue polypeptide and exists as a membrane-anchored precursor protein modified by myristoyl or palmitoyl adducts near its amino-terminus in keratinocytes (Chakravarty and Rice, 1989; Steinert *et al*, 1996b). Upon the terminal differentiation of keratinocytes, TGase1 is cleaved at two sites, which leads to a more active form (Rice *et al*, 1990; Kim *et al*, 1995a; Steinert *et al*, 1996a; Candi *et al*, 1998). The cleavage sites have been proved to be at the amino-terminal sides of glycyl resides at position 94 (Glycine 94) and at position 574 (Glycine 574) (Kim *et al*, 1995a). It is hypothesized that cleavage at Glycine 574 plays a part in removing the hindrance of catalytic core domain in the precursor form (Candi *et al*, 1998), or in enhancing the activity of active TGase1 fragment when they associate together (Kim *et al*, 1995a). The activation mechanisms are not well understood, however, especially *in vivo*, due to few useful tools with which the catalytic fragments of TGase1 can be detected directly in the epidermis.

We have successfully used cleavage site-directed antibodies in the past for various bioactive molecules (Imajoh-Ohmi *et al*, 1992; Kikuchi and Imajoh-Ohmi, 1995; Kato *et al*, 2000; Niikura

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Abbreviations: 5-BAP, 5-(Biotinamido) pentylamine; BSA, bovine serum albumin; CE, cornified envelope; LSE, living skin equivalents; PBS, phosphate-buffered saline (pH 7.4) without $CaCl_2$ and $MgCl_2$; RA, all-*trans* retinoic acid; TGase1, transglutaminase 1; TRITC, tetramethyl rhodamine isothiocyanate; VD, 1-α-25-dihydroxycholecalciferol.

et al, 2002). Therefore, in our new attempt to clarify the mechanism of activation and physiologic function of TGase1, we established two hybridoma cell lines producing such antibodies. One detects the amino-terminus of the fragment produced by cleavage at Glycine 94 (94G fragment), and the other detects the amino-terminus of the fragment produced by cleavage at Glycine 574 (574G fragment). As shown in this study, these antibodies provide us with novel tools to dissect the mechanism by which TGase1 is activated during the terminal differentiation of keratinocytes and the fate of TGase1-derived fragments in the construction of the epidermis. All experiments were performed with the approval of the Ethical Committee for Human Tissue Experiments of the Yakult Central Institute, and animals were handled according to the guidelines of the Ethical Committee for Animal Experiments of Yakult Central Institute.

MATERIALS AND METHODS

Establishment of hybridoma cell lines producing monoclonal antibodies Peptides corresponding to the amino-terminus of a fragment generated by cleavage at Glycine 94 (GSGVNAAGDGTIREG, Pep2) and its preprocessed form (CSDSRRPVSRGSGVNAAGDGTIREG, Pep1), or to the amino-terminus of a fragment generated by cleavage at Glycine 574 (GSAEDVAMQVEA, Pep4) and its preprocessed form (CSKPNVYANRGSAEDVAMQVEA, Pep3), were synthesized on a Shimadzu PSSM-8 peptide synthesizer according to the Fmoc method, and protecting groups and linkers were cleaved by treatment with trifluoroacetic acid. The peptides were then conjugated to a carrier protein (keyhole limpet hemocyanin) as previously described (Imajoh-Ohmi *et al*, 1992).

Female BALB/C mice were injected subdermally with 100 μg of the synthetic peptide (Pep2 or Pep4) emulsified with Freund's complete adjuvant (DIFCO, Detroit, Michigan) on day 1. Five additional immunizations with 100 μg of peptide were made intraperitoneally with Freund's incomplete adjuvant (DIFCO) at 2 wk intervals. The final injection of 100 μg of peptide in saline was made intravenously without adjuvant 5 d prior to being euthanized.

Spleen cells from the immunized mice were fused with mouse myeloma P3-NS-1/a-Ag4-1 cells (Mike *et al*, 1994), and the standard hybridoma technique was adopted. Briefly, after incubation for 5 min at 37°C in polyethylene glycol 6000 (Wako, Osako, Japan) containing 45% RPMI 1640 (Sigma, St. Louis Missouri), fused cells were grown in a selection medium consisting of RPMI1640 supplemented with 15% (v/v) fetal bovine serum (Dainippon Pharmaceutical, Osaka, Japan), 80 U per mL of penicillin G, 80 μg streptomycin per mL (Life Technologies, Rockville, Maryland), 0.1 mM hypoxamine, 0.4 μM aminopterin, and 0.016 mM thymidine at 37°C under a humidified atmosphere of 7% CO_2. Fourteen days after the fusion, cell supernatants were screened with an enzyme-linked immunosorbent assay using the respective synthetic peptides as immunogens. Cells giving positive signals were subcloned at least twice by the limited dilution method, and the antibodies were isotyped using a Mouse MonoAB ID/SP Kit (Zymed Laboratories, Inc., San Francisco, California). The hybridoma clone R1410H, obtained with the Pep2, produced IgG2a antibodies (anti-94G), whereas the hybridoma clone M13E, obtained with the Pep4, produced IgG2b antibodies (anti-574G). To obtain plenty of these monoclonal antibodies, hybridoma cells were injected intraperitoneally into mice, and the ascites fluids gathered were applied on protein G columns (Pharmacia Biotech, Uppsala, Sweden) to purify the antibodies.

Enzyme-linked immunosorbent assay enzyme-linked immunosorbent assay was carried out according to the method of Mike *et al* (1994) with slight modification. Briefly, each of the peptides (50 ng) was coated on immuno-plates (MaxiSorp from Nunc, Roskilde, Denmark) as immunogens. After blocking with bovine serum albumin fraction V (BSA; Boehringer Mannheim, Germany), the plates were incubated at room temperature for 1 h with purified antibodies at appropriate dilutions (1:2000). Peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, Pennsylvania) were added, and the plates were incubated at room temperature for 1 h, followed by an enzymatic reaction with the substrates for peroxidase (ImmunoPure TMB Substrate Kit; Pierce, Rockford, Illinois) at room temperature for 30 min. The reaction was terminated by the addition of 2 M sulfuric acid, and the absorbance at 492 nm was determined (Bio Kinetics Reader from Bio-Tek, Winooski, Vermont).

Preparation of monolayer-cultured keratinocyte Primary cultured normal human epidermal keratinocytes (Epidercell NHEK(F) from Kurabo Biomedical Business, Osaka, Japan) were seeded on tissue culture dishes (Becton Dickinson, Franklin Lakes, New Jersey) or collagen-coated cover glasses (Iwaki, Osaka, Japan) and grown at 37°C (5% CO_2, humidified atmosphere) in keratinocyte growth medium supplemented with 10 μg insulin per mL, 0.1 ng human epidermal growth factor per mL, 0.5 μg hydrocortisone per mL, 0.4% (v/v) bovine pituitary extract, 50 μg gentamicin per mL, and 50 ng amphotericin B per mL (Humedia-KG2; Kurabo Biomedical Business). For coercive differentiation, subconfluent cells were challenged with 1 mM $CaCl_2$ and incubated for 48 h.

Western blot and immunoprecipitation analyses For western blot analysis, 10^6 monolayer-cultured keratinocytes were harvested and sonicated in phosphate-buffered saline (pH 7.4) without $CaCl_2$ and $MgCl_2$ (PBS) containing an excess of protease inhibitor cocktail (Complete, Mini; Roche, Mannheim, Germany). After removing cellular debris by centrifugation (16,000 × *g*), the cell lysates were dissolved in sodium dodecyl sulfate (SDS) sample buffer containing 2.3% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, and 10 μg bromophenol blue per mL in 125 mM Tris–HCl (pH 6.8), and heated to 100°C for 3 min. These samples were subjected to SDS–polyacrylamide gel electrophoresis, 12% (w/v) acrylamide followed by electrical transfer on to a polyvinylidene fluoride membrane (Immobilon; Millipore, Billerica, Massachusetts). The membrane was soaked in Tris-buffered saline (pH 7.5) containing 20 mg BSA per mL for 1 h at room temperature, and incubated with anti-574G (1:1000 dilutions) for 2 h at room temperature. Then the antibody-treated membrane was washed to remove the excess antibodies, and incubated with anti-mouse IgG conjugated with alkaline phosphatase (Promega, Madison, Wisconsin). Antigens were visualized by the enzymatic reaction of alkaline phosphatase with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium (Promega).

For immunoprecipitation analysis, 10^7 monolayer-cultured keratinocytes were harvested. Immunoprecipitation with anti-94G (1:50 dilutions) was performed by using IMMUNOcatcher system (CytoSignal, Irvine, California). Pre-immune serum of normal BALB/C mouse was used to remove the components, which nonspecifically bind to antibodies. Protein specifically bound to anti-94G was subjected to SDS–polyacrylamide gel electrophoresis (7.5% (w/v) acrylamide) and detected by Coomassie brilliant blue staining.

Immunofluorescence analysis Frozen sections of normal human skin (BioChain Institute, Inc., Hayward, California), monolayer-cultured keratinocytes on collagen-coated cover glasses, and frozen sections of three-dimensional living skin equivalent (described below), were fixed with acetone at –20°C, rinsed with PBS containing 0.1% (w/v) BSA, and treated with 10% (v/v) normal horse serum (Boehringer Mannheim) to block nonspecific binding. Then the samples were incubated at room temperature for 2 h with the antibodies (1:50 dilutions). To perform epitope-competition experiments, antibodies were incubated with respective immunogens or control peptide at 4°C for 2 h before the sample incubation. Excess antibodies were removed, and the samples were again incubated at room temperature for 2 h with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Jackson ImmunoResearch). For some analyses, nuclei were stained with 10 μg propidium iodide per mL. These samples were mounted with mounting medium (Vectashield, Vector, Burlingame. California), and observed under a Confocal Laser Imaging System (Bio-Rad, Hercules, California). For the analysis of the epitope-competition experiments, FITC intensity was measured using an image analyzer with Win ROOF software (Mitani Corporation, Tokyo, Japan).

***In situ* TGase assays** Frozen sections of normal human skin (BioChain Institute, Hayward, CA) were rinsed with 1% (w/v) BSA in Tris-buffered saline, and incubated at 37°C for 1 h with 500 μM 5-(Biotinamido) pentylamine (5-BAP; Pierce, Rockford, Illinois) in Tris-buffered saline containing protease inhibitors (Complete, Mini, ethylenediamine tetraacetic acid-free, Roche, Mannheim, Germany) with 5 mM $CaCl_2$ or with 5 mM ethylenediamine tetraacetic acid. The sections were fixed with acetone at –20°C, rinsed with PBS, and treated with 10% (v/v) normal horse serum to block nonspecific binding. After incubation at room temperature for 1 h with TRITC-conjugated streptavidin (Jackson ImmunoResearch), the sections were mounted with mounting medium and observed with the Confocal Laser Imaging System (Bio-Rad).

Preparation of three-dimensional living skin equivalents (LSE) Three-dimensional LSE (manufactured by Toyobo, Osaka, Japan) were cultured at 37°C (5% CO_2, humidified atmosphere) in 1:1 HamF12/

Dulbecco minimal Eagle's medium containing 1% fetal bovine serum (LSE assay medium; Toyobo) by the air–liquid interface method (Bell *et al*, 1991). Some LSE were challenged with 5×10^{-7} M 1-α-25-dihydroxycholecalciferol (Solvay Pharmaceuticals, Weesp, the Netherlands) or 5×10^{-7} M all-*trans* retinoic acid (RA; Sigma) via the liquid phase (culture medium) at 2 wk after the beginning of the air–liquid interface culture, and then incubated for 4 d at 37°C. Other LSE were exposed to 25 mJ per cm^2 per day of ultraviolet B (VL-115 from Vilber Lourmat, Cedek, France; Ultraviolet spectral curve was measured by Spectroradiometer SS-11 from Maki Manufacturing Co. Ltd, Tokyo, Japan) via the air phase (stratum corneum equivalent of LSE) for 3 d, 2 wk after the beginning of the air–liquid interface culture. Immunofluorescence analyses of frozen sections of these LSE were performed similarly to those of normal human skin.

RESULTS

Preparation and specificities of cleavage site-directed antibodies for cleaved TGase1 We established two different hybridoma cell lines that produced antibodies against synthetic peptides corresponding to the two cleavage sites (**Figs 1** and **2*A***). In enzyme-linked immunosorbent assay, the antibody raised against Pep2 (anti-94G) exclusively bound to Pep2, and not to Pep1, 3, or 4 (**Fig 2*B***). Similarly, the antibody raised against Pep4 (anti-574G) predominantly bound to Pep4, with much less binding to Pep1, 2, and 3 (**Fig 2*C***).

In immunofluorescence analyses, these antibodies were successfully used to stain human epidermal sections as well as cultured human keratinocytes as described below (see Figs 3–5). We performed competitive inhibition experiments using these antibodies and synthetic peptides in these analyses. As shown in **Fig 2(*D*,*E*)** the immunofluorescence stainings with anti-94G and anti-574G were clearly inhibited by prior incubation of the antibodies with Pep2 and Pep4, respectively, but not with the corresponding precursor peptides (Pep1 and Pep3).

In order to detect the binding of these antibodies to cellular TGase1-derived fragments, we performed western blot analysis of monolayer-cultured keratinocyte lysates. Anti-574G detected 33 kDa protein in the cytosol fraction of the cell. Furthermore, the intensity of the protein band increased as cell differentiation was induced with Ca^{2+} (**Fig 2*F***). On the other hand, we could not detect any specific protein band with anti-94G in the same western blot analysis (data not shown). From the consideration that the preparation method for western blot would severely reduce the antigenic property of the antigen of anti-94G, immunoprecipitation assay was performed. Consequently, anti-94G specifically bound to 67 kDa protein in coercively differentiated keratinocyte lysates (**Fig 2*G***).

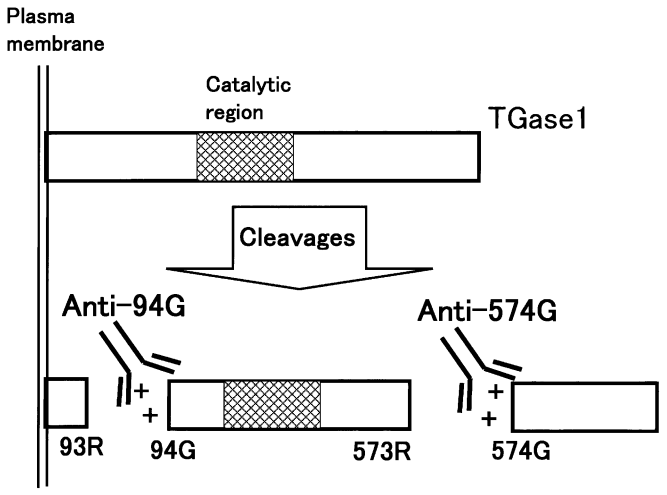

Figure 1. Cleavages of TGase1 and cleavage site-directed antibodies. The carboxyl-terminus of TGase1 is anchored to the plasma membrane. The meshed portion indicates the catalytic region for TGase cross-linking activity. The intact TGase1 is cleaved (Cleavages) between arginine 93 (93R) and glycine 94 (94G), or between arginine 573 (573R) and glycine 574 (574G), and catalytic fragments are produced. Cleavage site-directed antibodies (anti-94G, anti-574G) detect a positive charge (+) in the amino-terminus (94G, 574G) of the newly produced catalytic fragments.

Immunohistochemical analysis of normal human skin epidermis **Figure 3** shows immunofluorescence microscopy of a typical sample of human skin stained with each of the monoclonal antibodies. Most of the epidermal layers were stained with both anti-94G and anti-574G. When looking into the stained regions in more detail, however, there are substantial differences in the staining patterns between them. Anti-574G stained the entire epidermis, especially the basement membrane and stratum corneum, whereas the regions stained with anti-94G included the suprabasal layer, spinous layer, and granular layer, but not the basement membrane or stratum corneum. In addition, the anti-94G-positive regions were limited to the periphery of each keratinocyte, whereas anti-574G stained the cytosolic regions of the cells. Hair follicles were also stained by both anti-574G and anti-94G.

Similar staining patterns were observed in 16 normal human skin samples (data not shown).

TGase cross-linking activity in epidermis We measured the cross-linking activity of TGase in epidermis by detecting 5-BAP (Lee *et al*, 1988) incorporation as described in *Materials and Methods*. Consequently, 5-BAP was incorporated into the entire epidermis (**Fig 6*A***). As 5-BAP is not a specific substrate for TGase1, there is a possibility that we also detected the activity of TGase3 in the granular layer, with the result that a much greater extent of incorporation in the granular layer than in the other layers was detected; however, because pH 7.4 assay conditions in our experiments are more suitable for the detection of activity derived from TGase1 than from TGase3 (Raghunath *et al*, 1998), and as no other TGase isotype has been described in the region, the activity detected in the basal and spinous layers cells is probably derived from TGase1. The 5-BAP incorporated into the region was localized to the periphery of the keratinocytes (**Fig 6*B***), which coincided with the localization pattern of the antigen detected by anti-94G (**Fig 3*B***). In the absence of Ca^{2+}, 5-BAP was not incorporated into the epidermis (**Fig 6*C***).

Detection of TGase1 fragments in monolayer-cultured keratinocytes At low Ca^{2+} concentrations in the culture medium (0.03–0.3 mM), primary cultured keratinocytes grow as a monolayer with a high rate of proliferation until they become confluent. Postconfluently cultured cells differentiate spontaneously even at low Ca^{2+} concentrations (Dlugosz and Yuspa, 1994); when the Ca^{2+} concentration in the medium is raised to 1.8 mM, cell differentiation is induced more coercively, resulting in the expression of many differentiation markers, including TGase1 (Hennings *et al*, 1989; Dlugosz and Yuspa, 1994; Gibson *et al*, 1996). We employed these cultured cells to investigate the relationship between cell differentiation and the cleavage of TGase1.

Normal human primary keratinocytes were seeded and grown in the growth medium (0.3 mM $CaCl_2$) until they became confluent. Aliquots of the cells were cultured postconfluently to induce differentiation spontaneously, or treated with 1 mM $CaCl_2$ to induce differentiation coercively.

The anti-574G stained both highly proliferating cells (**Fig 4*A***) and confluent cells (**Fig 4*B***). The staining was detected mostly in the cytosol, and was enhanced in differentiating cells (**Figs 2*F*** and **4*C*,*D***).

In contrast to the results with anti-574G, the anti-94G antibody stained only differentiating cells (**Figs 2*G*** and **4*G*,*H***), rather than

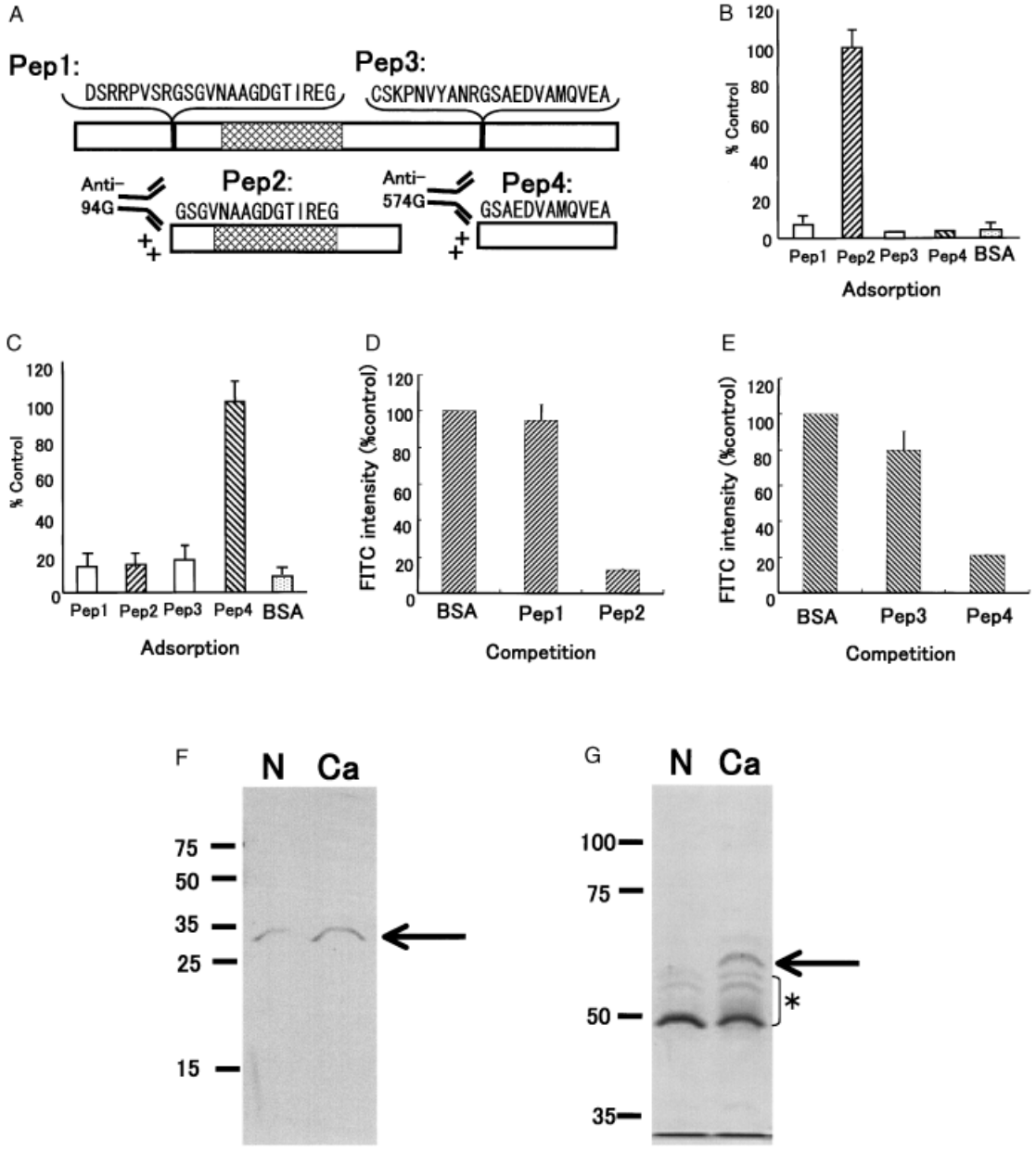

Figure 2. Specificity of Anti-94G and Anti-574G. Synthetic peptides mimicking the amino-terminus of each catalytic fragment (Pep2, Pep4) were used as immunogens in the preparation of anti-94G and anti-574G. Pep1 and Pep3 indicate synthetic peptides mimicking the uncleaved forms (*A*). Anti-94G was predominantly adsorbed by Pep2 (*B*), whereas anti-574G was predominantly adsorbed by Pep4 (*C*) on an enzyme-linked immunosorbent assay. Anti-94G was adsorbed by Pep2 rather than Pep1 on immunohistochemical staining (*D*), whereas anti-574G was adsorbed by Pep4 rather than Pep3 (*E*). Error bars: mean $\pm$ SD ($n = 6$). (*F*) Unchallenged subconfluent cells (N), and subconfluent cells challenged with 1 mM $CaCl_2$ (Ca) were cultured for 48 h, and prepared for a western blot using anti-574G as described in *Materials and Methods*. Lines with numbers at the left indicate molecular markers (kDa). ←, Anti-574G bound to 33 kDa protein. (*G*) Unchallenged subconfluent cells (N), and subconfluent cells challenged with 1 mM $CaCl_2$ (Ca) were cultured for 48 h, and prepared for immunoprecipitation analysis using anti-94G as described in *Materials and Methods*. Lines with numbers at the left indicate molecular markers (kDa). ←, Anti-94G bound to 67 kDa protein. Heavy chain of anti-94G and other protein corresponding to anti-94G are indicated with an asterisk.

the proliferating (**Fig 4*E***) or confluent cells (**Fig 4*F***). The staining was detected mostly in the cell periphery.

Immunofluorescence analysis of three-dimensionally differentiating cells When normal primary keratinocytes are cultured by the air–liquid interface method on collagen lattices containing dermal fibroblasts, they differentiate three-dimensionally until a stratum corneum-like structure is formed (Bell *et al*, 1991). This three-dimensional culture, termed LSE, shows a high degree of similarity to normal human epidermis in morphologic and biochemical characteristics (Bilbo *et al*, 1993; Nolte *et al*, 1993).

When a normally cultured LSE was stained with either anti-94G or anti-574G, the staining pattern was quite similar to that of normal human epidermis (**Fig 5*A*,*E***). Addition of 1-α-25-dihydroxycholecalciferol (VD) to the culture medium slightly increased the intensity of staining by both anti-94G and anti-574G. The epidermis equivalent was slightly hyperplastic (**Fig 5*B*,*F***). Addition of RA to the culture medium decreased the intensity of staining with anti-94G, especially at the periphery of keratinocytes in the basal layer equivalent, whereas staining with anti-574G seemed not to be significantly affected. The cells became squamous (**Fig 5*C*,*G***). Exposure to ultraviolet B clearly reduced the intensity of staining not only by anti-94G but also

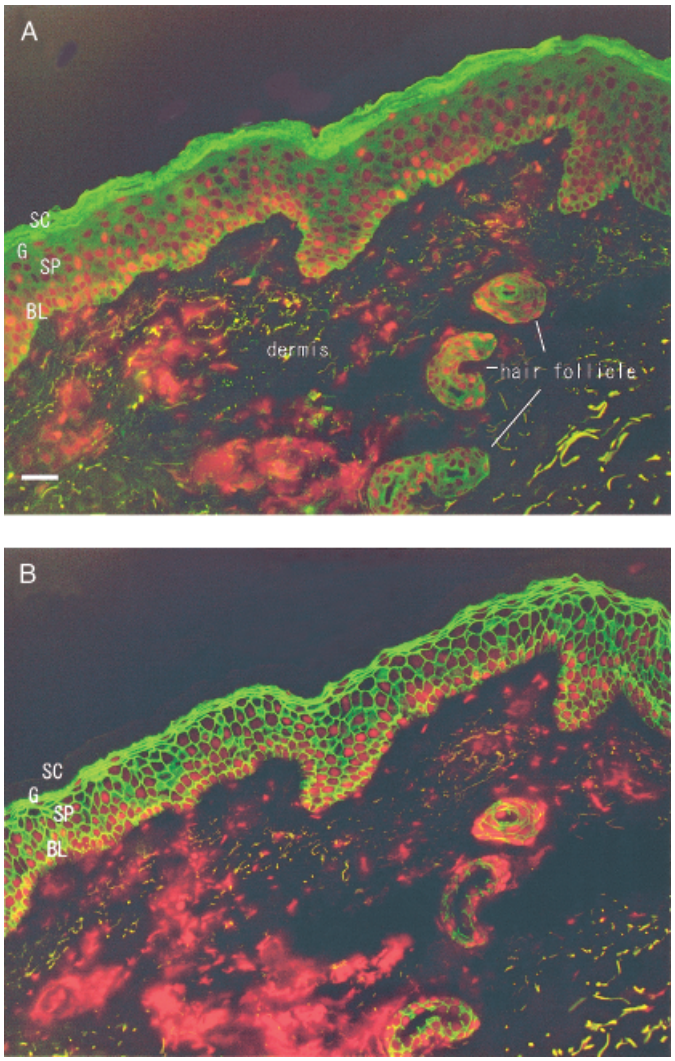

Figure 3. Immunofluorescence analysis of normal human skin. Frozen sections of normal human skin were incubated with anti-574G (*A*) or anti-94G (*B*), and visualized using FITC (green) as described in *Materials and Methods*. Nuclei were stained with propidium iodide (red). *Scale bar* = 50 μm. SC, stratum corneum; G, granular layers; SP, spinous layers; BL, basal layers.

by anti-574G. The cells swelled, resulting in modulation of the morphology of the epidermis equivalent (**Fig 5*D*,*H***).

DISCUSSION

Cleavage site-directed antibodies and their specificities The TGase1 protein is known to be very unstable, and is thus difficult to purify in quantity as an immunogen for the preparation of antibodies. To detect proteolytically processed TGase1 proteins in human epidermis, we therefore employed synthetic peptides that mimic the cleavage sites of TGase1 as immunogens, and used them to establish hybridomas producing TGase1-specific monoclonal antibodies (**Figs 1** and **2*A***).

The binding specificities of these monoclonal antibodies against immunogen peptide are obvious from enzyme-linked immunosorbent assay (**Fig 2*B*,*C***). In addition, these antibodies bind to cellular components of proliferating and/or differentiating keratinocytes in immunofluorescence analyses (**Figs 3–5**). The bindings of which were much reduced by the prior incubation of the antibodies with the corresponding

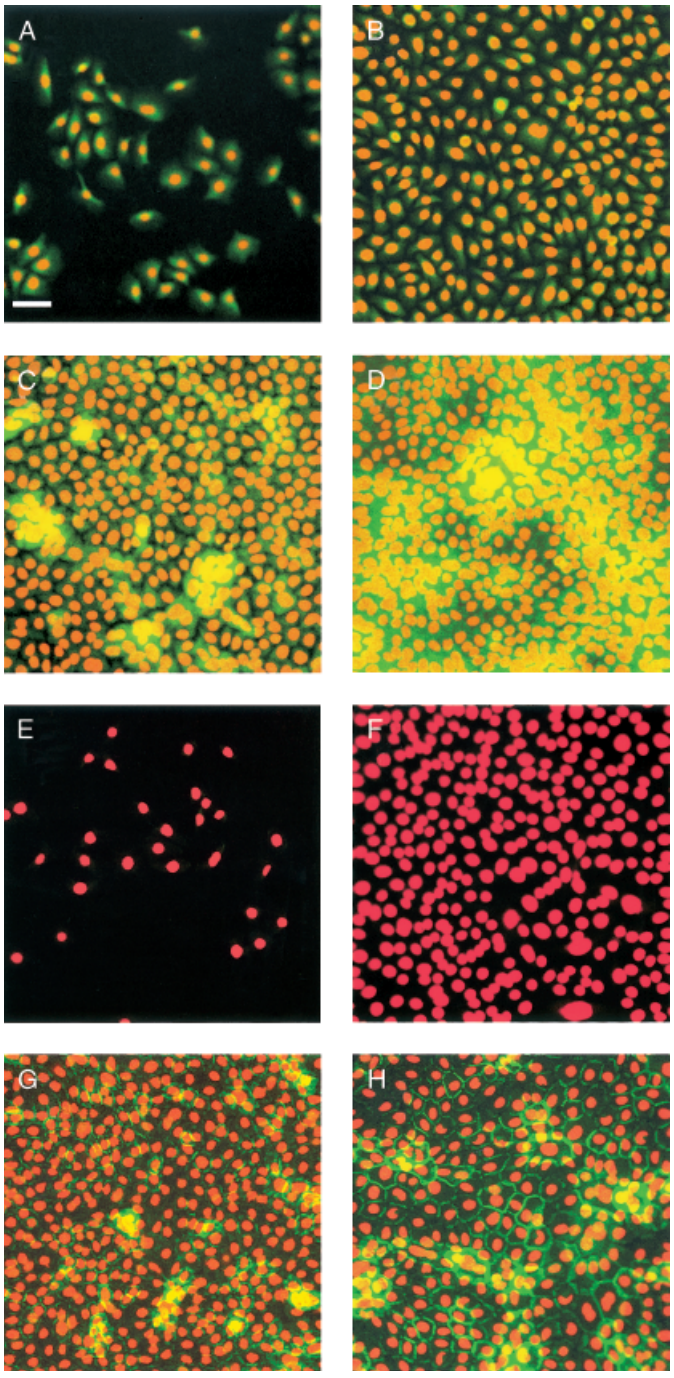

Figure 4. Immunofluorescence analysis of monolayer cultured cells. Normal human epidermal keratinocytes were seeded and grown in 0.3 mM calcium. Cells 1 d after the seeding (*A*,*E*), confluent cells (*B*,*F*), cells postconfluently cultured for 48 h (*C*,*G*), and cells challenged with 1 mM $CaCl_2$ at subconfluent and cultured for 48 h (*D*,*H*) were stained with anti-574G (*A–D*) or anti-94G (*E–H*), and visualized using FITC (green) as described in *Materials and Methods*. Nuclei were stained with propidium iodide (red). *Scale bar* = 50 μm.

immunogen peptides (**Fig 2*D*,*E***). Moreover, western bolt analysis for anti-574G and immunoprecipitation analysis for 94G gave unique protein bands of 33 kDa and 67 kDa in molecular weight, respectively (**Fig 2*F*,*G***). These values are consistent with those reported previously (Kim *et al*, 1995a). Therefore, we conclude that the monoclonal antibodies established in this

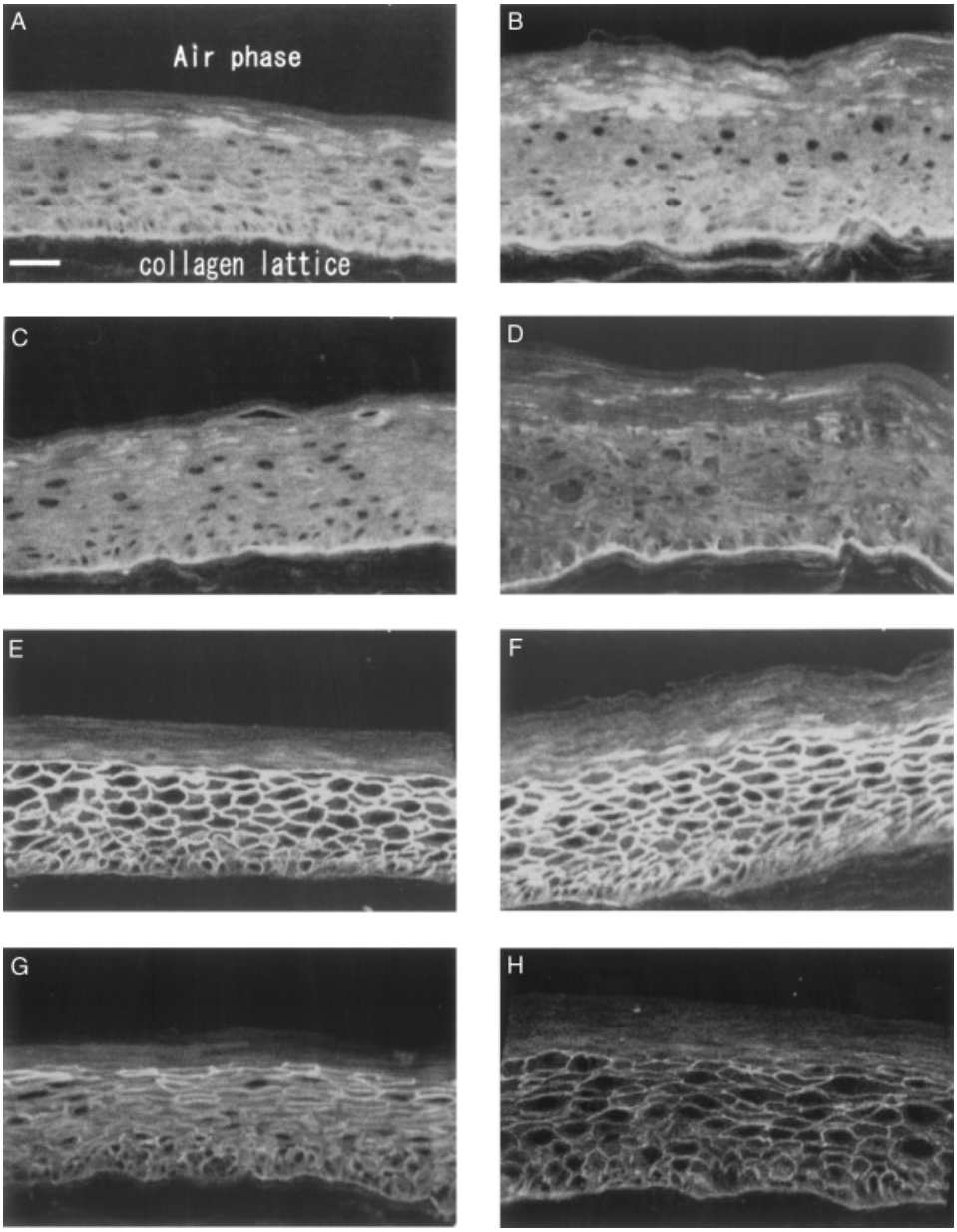

Figure 5. Immunofluorescence analysis of three-dimensional skin equivalents. LSE were cultured for 4 d without (*A,E*) or with 5×10^{-7} M 1-α-25-dihydroxycholecalciferol (*B,F*) or 5×10^{-7} M RA (*C,G*). Others were exposed to 25 mJ per cm^2 ultraviolet B radiation from the air phase every 4 d (*D,H*). The LSE were stained with anti-574G (*A–D*) or anti-94G (*E–H*) and visualized using FITC as described in *Materials and Methods. Scale bar* = 50 μm.

work bind specifically to native TGase1-derived fragments. We do not know why anti-94G could not bind to 67 kDa protein in western blot analysis. It may be possible that treatment with the SDS sample buffer and/or polyvinylidene fluoride membrane attachment change the immunologic property of the protein.

Cleavage occurs in the basal layer cells of human epidermis As shown in this study, both anti-94G and anti-574G detected the TGase1 fragments early in the terminal differentiation of keratinocytes in the basal layer, as well as in the whole human epidermis (**Fig 3*A*,*B***). Furthermore, the results of our *in situ* TGase assays on normal human skin suggested that 94G fragment detected in the basal layer keratinocytes already have enzymatic activity (**Fig 6*B***).

It has been thought that TGase1 protein is expressed mainly in the granular layer and plays a part in construction of the CE; however, considering some previous reports that suggest TGase1 protein exists not only in granular layer but also in suprabasal and spinous layers (Kim *et al*, 1995b), the subcellular localization and function of TGase1 need to be re-evaluated. Recently, TGase1 was shown to be concentrated at E-cadherin-based adherence junctions in mouse liver, suggesting that the TGase1 cross-linking activity is important for the maintenance of the structural integrity of simple epithelial cells (Hiiragi *et al*, 1999). This report supports the idea that the TGase1 cross-linking activity is not only used in the formation of CE in the granular layer, but also in some other physiologic function, especially in basal and spinous layer cells.

Cleavage at Glycine 574 precedes the cleavage at Glycine 94 On monolayer-cultured cells, 574G fragments were detected in highly proliferative cells (**Fig 4*A***) as well as in differentiating cells (**Fig 4*C*,*D***), whereas 94G fragments were confined to differentiating cells (**Fig 4*G*,*H***). These results suggest that the cleavage at Glycine 574 takes place earlier than that at Glycine 94, even in low concentration of Ca^{2+} in the medium, implying that two kinds of proteases may be involved in the cleavage of TGase1, or at least, that two cleavage events are controlled differently by a single protease. Although calpain, a Ca^{2+} dependent intracellular protease, is a candidate of these proteases (Garach-Jehoshua *et al*, 1998; Kim and Bae, 1998), the effect of various protease inhibitors and biochemical reagents to

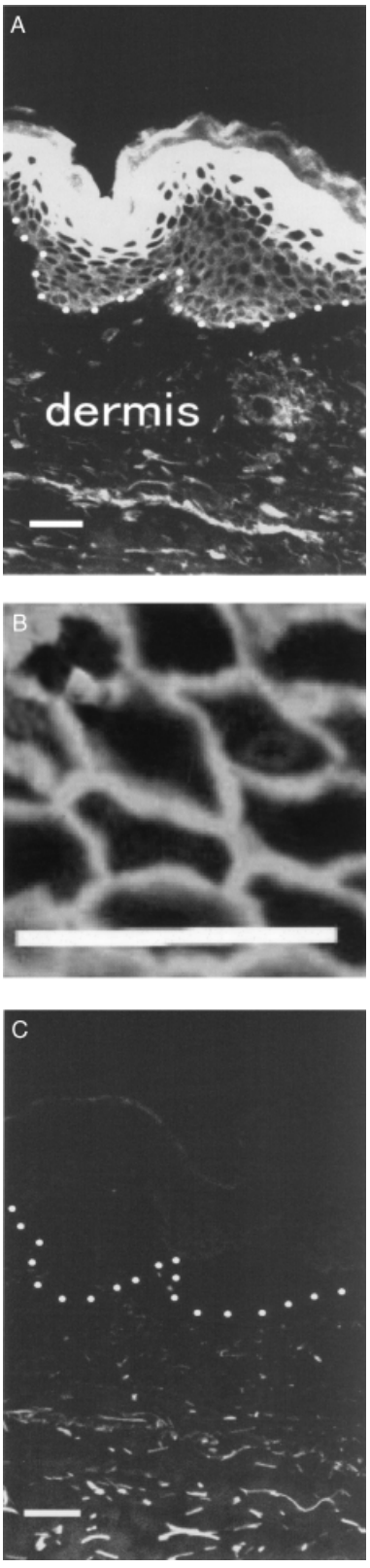

Figure 6. ***In situ*** **TGase assays of normal human skin.** 5-BAP incorporation with 5 mM $CaCl_2$ (*A,B*) or with 5 mM ethylenediamine tetraacetic acid (*C*) in normal human skin was visualized using TRITC as described in *Materials and Methods*. Positions of basement membranes are shown by dotted lines (*A,C*). (*B*) Expansion chart of suprabasal layer. *Scale bar* = 50 m.

regulate subtle changes in the intracellular Ca^{2+} concentration (e.g., Ca^{2+} ionophores) may provide us with additional information on proteolytic activation of TGase1.

Fate of 94G and 574G fragments In this study, the 94G fragment was mainly detected along the periphery of keratinocytes on immunohistochemical analysis (**Figs 3*B*** and **4**). These results imply that the 94G fragment is localized at the plasma membrane. Consistent with this result, some previous reports describe that the binding to membranes directs activity of TGase1 (Steinert *et al*, 1996b; Nemes *et al*, 1999a, 2000). An interesting question arising is what makes the 94G fragment remain at the plasma membrane despite of the amino-terminal region for membrane anchoring. In the basement membrane, 94G fragments were not detected (**Fig 3*B***). The acceptor of 94G fragment might be deficient in the plasma membrane adjacent to basement membrane.

In contrast to the 94G fragment, the 574G fragment, which has no enzymatic activity, is localized to the cytosolic portion of the epidermis. Furthermore, the 574G fragment accumulates in the stratum corneum, where the 94G fragment is not detected (**Fig 3*A***). This can be explained in that the 574G fragment is stably maintained throughout the course of cell differentiation, independently to the 94G fragment. Therefore, the 574G fragment may have another function in the process of normal epidermis biogenesis, in addition to the previously hypothesized role in removing the hindrance of catalytic core domain of its own precursor formation (Candi *et al*, 1998), and in increasing the activity of 94G fragment when they associate together (Kim *et al*, 1995a).

A novel approach to detect the function of TGase1 in various conditions We were successful in detecting TGase1 fragments in LSE as well. This *in vitro* system enabled us to detect possible changes in epidermis of TGase1 processing and subcellular localization of the fragments after various treatments. Treatment with VD, a possible activator of TGase1 in cultured keratinocytes (Claus *et al*, 2000), RA, an inhibitor of TGase1 activity in cultured keratinocytes (Fisher and Voorhees, 1996), or ultraviolet B irradiation modulates generation of the TGase1 fragment and/or localization of the fragments. Furthermore, the morphology of terminally differentiated keratinocytes were strongly affected when the localization of TGase1 fragments were disturbed, although we do not know yet whether these two events are related to each other (**Fig 5**).

RA is known as one of the bioactive reagents that causes complex phenomenon to keratinocyte. Saunders *et al* (1993) reported that TGase1 mRNA levels are downregulated in RA-treated cultured cells, whereas the most striking effects of RA treatment observed in our experiments were the disturbance of subcellular localization of the 94G fragment, although expression levels of TGase1 mRNA could not determined. In our opinion, from this result, the altered biogenesis of epidermis by treatment with RA may be caused at least in part by abnormal subcellular localization of the active TGase1 fragment. As such, our new antibodies will be useful tools for investigations about proteolysis and/or localization of cellular TGase1 proteins in various conditions.

In conclusion, we have established mouse hybridomas that produce cleavage site-specific antibodies against human TGase1 fragments. The antibodies allow us to dissect the mechanism by which TGase1 is activated and provide us with novel insights into the biogenesis of epidermis.

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