Transdifferentiation of Epidermis to Mucous Epithelium by Retinol Accompanies Increase in Transglutaminase 2/Gh and Decrease in Transglutaminase 3

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We showed previously that transdifferentiation of skin epidermis to mucous epithelium can be induced by treatment with 20 μM retinol for 1 d followed by culture for 4 d without retinol in chick embryonic tarsometatarsal skin. In mouse epidermal cells, 3 μM retinoic acid (an active metabolite of retinol) inhibits epidermal keratinization in consistent with an increase in transglutaminase (TG)/2/Gh, while its physiological role in the skin is still unresolved. TG1, TG3 and TG5 are also found in mammalian keratinocytes and play an important role in the formation of the stratum corneum in the skin by the introduction of cross-links into proteins. The most characteristic enzyme function of TG family is calcium-dependent transamidation activity (transamidase) that introduces inter or intramolecular ε-(γ-glutamyl)lysine cross-links into the protein. TG2/Gh is a multifunctional protein and ubiquitously expressed member of transglutaminase family that has been implicated in a variety of biological processes. By in situ hybridization analysis, we showed that TG2/Gh mRNA expression started to increase throughout the skin during the culture for 1 d with retinol, while it was weak in the control skin. On the other hand, an expression of TG3 mRNA was increased in the keratinized epidermis of control skin but was decreased by retinol. In situ transamidase activity of transglutaminase was weak in retinol-pretreated skin. Therefore, it was indicated that functions other than transamidase of TG2/Gh protein might be important in retinol-induced epidermal mucous transdifferentiation.

Key words: retinol; retinoic acid; transglutaminase 2; transglutaminase 3; skin; mucous transdifferentiation

Epithelial-mesenchymal interactions are important in development, cellular growth and differentiation both during embryogenesis and in adult animals, including reproduction.1 Retinoids function as important regulatory signaling molecules in these processes2; in our previous studies, we showed that transdifferentiation of epidermis to mucous epithelium (epidermal mucous metaplasia) of chick embryonic tarsometatarsal skin can be induced by culture of recombinant of undifferentiated epidermis with excess retinol-pretreated dermis,3 and that the signal(s) for mucous transdifferentiation, which was induced in the dermis during the first 8 h of culture by retinol, requires protein synthesis,4 not protein glycosylation,4 although the signal molecule is still unknown.

Retinoic acid at a concentration of 3 μM increased an expression of transglutaminase (TG)/2/Gh after culture for 24 h in rat epidermal cell, while its physiological role in the skin is still unresolved.5 The most characteristic enzyme function of the class of enzymes known as TG is the formation of covalent bonds between epsilon amino groups of primary amines (lysine or others) and the gamma-carboxyamine group of glutamine residues of proteins, resulting polymerization.5 Transglutaminase 1, 3 and 5 found in the keratinocyte6 are members of transglutaminase family and play an important role in the formation of the stratum corneum in the epidermis of skin by the introduction of γ-glutamyl-ε-lysyl isopeptide bond cross-links into proteins.6 TG2/Gh, another type of transglutaminase, is a unique and the most diverse and ubiquitously expressed member of transglutaminase family of proteins. In addition to its calcium-dependent transamidation activity, it has also been reported to be a protein disulfide isomerase that regulates adenylate cyclase,7 to function as a signal-transducing guanosine 5′-triphosphate (GTP)-binding protein8 from classical G-coupled receptors, and to have serine/threonine kinase.9 TG2/Gh knockout mice do not have any defects in the keratinocyte differentiation program.10 TG2/Gh is involved in wound healing10 and its role in apoptosis remains unclear.11 TG2/Gh, which is localized primarily in the cytosol in a catalytically latent form,12 has the potential to translocate to the nucleus, to be externalized, and to ultimately co-localize with proteins in the extracellular matrix or on the extracellular side of the plasma membrane.12

Here, we studied whether the expression of TG2/Gh mRNA is increased by retinol and whether the transamidase activity of the enzyme is required for retinol-induced epidermal mucous metaplasia.

MATERIALS AND METHODS

Skin Culture Skin explants from the tarsometatarsal region of 13-d-old chick embryos that had been cultured for 1 d in a chemically defined medium, BGJb (Sigma), containing 5% delipidized fetal calf serum (dFCS)13 and 20 μM hydrocortisone hemisuccinate (Japan Upjohn Ltd., Tokyo) with or without 20 μM retinol (Sigma) were cultured for 4 d in BGJb containing 2 mM dibutyric cAMP (Sigma), which enhances retinol-induced epidermal mucous metaplasia14; the skin was cultured in a closed tube in the dark by the Millipore filter-roller-tube method.15 As 10—20 μM retinol, not 5 μM, could induce epidermal mucous metaplasia, 20 μM retinol was used in the experiment. Monodansylcadaverine (MDC) (Sigma) was used as a cell-permeable transglutaminase competitive inhibitor.16 In some cases, 40 μM MDC was added throughout the culture.

Preparation of Digoxigenin-Labeled TG2/Gh and TG3
RNA  Total RNA was isolated from the cornea of 15-d-old chick embryos using TRIZOL LS Reagent (Gibco BRL). After 1 μg of total RNA was reverse transcribed at 37 °C for 2 h with oligo dT30 and M-MLV reverse transcriptase (Gibco BRL), 100 ng cDNA was used for the polymerase chain reaction (PCR) for TG3 with Ampli Taq Gold DNA polymerase (Perkin Elmer). One hundred ng of DNA from the cDNA library of 5-d-old chick embryos was used as DNA template in the PCR reaction for TG2/Gh. The oligonucleotides synthesized for reverse transcriptase (RT)-PCR were derived from neighboring exons and were: 5'-ATGCGGACCCGAC-3' (TG2/Gh, sense), 5'-AGTGCGCACTGTGCC-3' (TG2/Gh, antisense), nucleotides 1–256 in TG2/Gh gene. The oligonucleotides synthesized with DIG-labeled probes was performed as described previously.17) The PCR steps included an initial denaturation at 95 °C for 12 min followed by a denaturing temperature of 95 °C for 1 min, an annealing temperature of 65 °C (TG2/Gh) and 51 °C (TG3) for 30 s, and an extension temperature of 72 °C for 1 min for 40 cycles. The PCR product of TG2/Gh, which was performed in the presence of 2% dimethyl sulfoxide (DMSO), was subcloned into pBluescriptSK(-). After digestion of the TG2/Gh pT7Blue T plasmid with BamHI (to produce antisense probe, Takara) or TG2/Gh pBluescriptSK(-) plasmid with XhoI (to produce sense probe), the linearized plasmid was transfected with T7 RNA polymerase in transcription reactions containing Digoxigenin-11-UTP (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's reagents and instructions. The PCR product of TG3 was subcloned into pT7Blue T-vector (Takara, Kusatsu, Shiga, Japan) and then sequenced. The subcloned TG2/Gh pT7BlueT plasmid was digested with XbaI (Takara) and KpnI and the obtained DNA fragment was further subcloned into pBluescriptSK(-). After digestion of the TG2/Gh pT7Blue T plasmid with BamHI (to produce antisense probe, Takara) or TG2/Gh pBluescriptSK(-) plasmid with XhoI (to produce sense probe), the linearized plasmid was transfected with T7 RNA polymerase for antisense or sense probe, respectively.

In Situ Hybridization  
In situ hybridization with the DIG-labeled probes was performed as described previously.17)

In Situ Transamidation Activity of TG  For TG substrates, 2 mM EZ-Link 5-(biotinamido) pentylamine (Pierce), was added to the culture medium during the last 2 h of culture.18) Frozen sections were cut at a 7-μm thickness from optimal cutting temperature (OCT) compound-embedded dorsal skin, mounted on 2% neoprene (Nisshin EM Co., Ltd.)-coated slides, and fixed in phosphate-buffered saline (PBS) containing 2% paraformaldehyde for 10 min. The sections were rinsed twice in PBS for 15 min each time, and were probed with streptavidin-conjugated horseradish peroxidase (Amersham Biosciences) at a dilution of 1:3000 at room temperature for 15 min. Enhanced chemiluminescence (ECL) Western blotting detection reagents and analysis system (Amersham Biosciences) was used for detection of immobilized streptavidin-conjugated horseradish peroxidase. Protein was determined in the absence of mercaptoethanol using a BCA Protein Assay Kit (Pierce). These experiments were performed according to the manufacturer's instructions. Animal experiments was conducted in accordance with the experimental animal guidelines of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

RESULTS

Increase in TG2/Gh mRNA Expression by Retinol in Cultured Skin  TG2/Gh mRNA was expressed in 13-d-old chick embryonic skin (Figs. 1A, B). When the skin that had been cultured in the presence of 20 nm hydrocortisone with 20 μM retinol for 1 d was cultured in the absence of hydrocortisone and retinol for additional 4 d, the strong expression of TG2/Gh was observed in the skin cultured for 1 d (Figs. 1C, D) and 5 d (Figs. 1G—J). The epidermis in retinol-pre-

Fig. 1.  In Situ Hybridization Analysis of the TG2/Gh Expression in the Cultured Skin

Arrowheads show boundary between epidermis and dermis. Thirteen-day-old chick embryonic skin (A, B) was cultured for 24 h in BFGm containing 20 nm hydrocortisone with (C, D, G—J) and without (E, F, K, L) 10 μM retinol and then in BFGm with 2 mM Br,2cAMP for 4 d. Antisense riboprobe (A, C, E, G, I, K), sense riboprobe (B, D, F, H, J, L). e, epidermis; d, dermis. Bar, 100 μm.
Suppression of TG3 Expression by Retinol in Cultured Skin

As skin keratinocytes express transglutaminase 1, 3 and 5, all of which are involved in the formation of cornified envelope by the introduction of γ-glutamyl-ε-lysyl isopeptide bond cross-links into the envelope protein, we assumed that retinol that suppressed epidermal keratinization might inhibit their expression. Hence we studied the expression of TG3 mRNA as representative of them in retinol-pretreated skin. In situ hybridization study showed that TG3 mRNA was only slightly expressed, if at all, in the undifferentiated epidermis of 13-day-old chick embryonic skin (Figs. 2A, B) and in the epidermis of retinol-induced mucous skin during culture for 1 or 5 d (Figs. 2C, D, G, H). In contrast, in control skin, it was induced to express in the epidermal basal and superficial layers after culture for 1 d (Figs. 2E, F) and the expression became stronger in the epidermal suprabasal layers after culture for 5 d (Figs. 2I, J).

In Situ Transamidation Activity of TG in Retinol-Pretreated Cultured Skin

Biotinylated pentaamine (BPNH2), which acts as an acyl-acceptor, enters living cells and acts as a tag that is cross-linked by TG to glutamine donor substrates. When the retinol-pretreated skin or control skin was cultured for 4 d and 2 mM BPNH2 was added during the last 1 h of the culture, incorporation of BPNH2 into the protein of the extra cellular or intracellular side of plasma membrane with low density was observed in the supra basal layers in retinol-pretreated skin (Figs. 3A, B). On the other hand the stronger expression was observed in the superficial layers of the epidermis with high density in control skin (Figs. 3C, D). The TG substrates in the dermis were little both in retinol-pretreated skin and control skin (Figs. 3A—D). When separating the protein on SDS-PAGE and probing blots with streptavidin-peroxidase, BPNH2 was incorporated into the numerous endogenous protein substrates of TG in the skin,

Fig. 2. In Situ Hybridization Analysis of the TG3 Expression in the Cultured Skin

Arrowheads show boundary between epidermis and dermis. Thirteen-day-old chick embryonic skin (A, B) was cultured for 24 h in BGJb containing 20 μM hydrocortisone with (C, D, G, H) and without (E, F, I, J) 10 μM retinol and then in BGJb with 2 mM Bt2cAMP for 4 d. Antisense riboprobe (A, C, E, G, I); sense riboprobe (B, D, F, H, J). c, epidermis; d, dermis. Bar, 100 μm.

Fig. 3. Identification and Fractionation of Acyl-Acceptor Transamidase Protein Substrates in the Skin

Thirteen-day-old chick embryonic skin was cultured for 24 h in BGJb containing 20 μM hydrocortisone with (A, B) and without (C, D) 10 μM retinol and then in BGJb with 2 mM Bt2cAMP for 4 d. Biotinylated substrates in the skin were stained with streptavidin-conjugated FITC (A, C). (B, D) Double-exposure images of luminescence of streptavidin-conjugated FITC (green) and DAPI (blue)-stained nuclei. Decrease and different localization of transamidase activity of transglutaminase in retinol-pretreated skin compared with that of control skin. Dotted lines show boundary between epidermis and dermis. (E) Biotinylated substrates in the skin were separated through standard SDS electrophoresis, and then were transferred to nitrocellulose membrane. The membrane was probed using streptavidin-conjugated horseradish peroxidase ECL Western blotting detection reagents and analysis system. Decrease in transamidation activity of TG in retinol-pretreated skin compared with that of control skin after culture for 5 d. There were few transamidation substrates of TG in the dermis of retinol-pretreated skin and control skin compared with those in the whole skin. The results shown are representative of three independent experiments.

Fig. 4. Effect of MDC on the Epidermis in Retinol-Pretreated Cultured Skin

Thirteen-day-old chick embryonic skin was cultured for 24 h in BGJb containing hydrocortisone with (A—D) and without (E—H) retinol and then in BGJb with Bt2cAMP for 4 d in the presence (C, D, G, H) or absence of MDC (A, B, E, F) throughout the culture. (A, C, E, G) Hematoxylin and eosin-stained sections. (B, D, F, H) Periodic acid-Schiff-stained sections.
treated skin and control skin were few with almost the same electrophoretic pattern (Fig. 3E). These results together indicated that, while TG2/Gh mRNA increased in retinol-pretreated skin, in situ transamidation activity of TG2/Gh was weak, indicating TG2/Gh functioned as other than transamidase.

**Effect of Monodansyl Cadaverine (MDC) on the Epidermis in Retinol-Pretreated Cultured Skin** The skin that had cultured with hydrocortisone in the presence or absence of retinol for 1 d was cultured for 4 d without hydrocortisone and retinol in the presence or absence of MDC at an optimal concentration of 40 μM throughout the culture. In the absence of MDC, mucous droplets appeared in the superficial layer of the epidermis (Figs. 4A, B). In the presence of MDC, mucous droplets in the epidermis disappeared in retinol-pretreated skin after culture for 5 d, while rounded cells characteristic of mucous cells appeared in the superficial layer of the epidermis or periderm (Figs. 4C, D). Periderm that was filled with peridermal granules was not affected in the presence or absence of MDC in control skin (Figs. 4E—H), presumably because the superficial layer was not cornified as yet, although flattened cells characteristic of differentiated cells were seen.

**DISCUSSION**

Retinoic acid, an active metabolite of retinol, is a consistent inducer of TG2/Gh expression. Calcium ion-dependent transamidase activity (TG2/Gh and TG3) can be measured in the presence of Ca²⁺ by in vitro transamidase activity. As strong mRNA expressions of TG2/Gh in retinol-pretreated skin and TG3 in control skin were observed, in vitro transamidase activity of retinol-pretreated skin would be expected to be the same as that of control skin. Hence, we studied about in situ transamidase activity to know the real enzyme activity in the epidermis. In this study, we showed that retinol suppressed expression of TG3 mRNA but increased expression of TG2/Gh mRNA, whose protein did not function as transamidase, if at all, because in situ transamidation activity was weak.

Furthermore, MDC, transglutaminase competitive inhibitor, did not inhibit the retinol-induced epidermal transdifferentiation, indicating again that transamidation activity of TG2/Gh might not be required for retinol-induced epidermal transdifferentiation to mucous epithelium, but TG3 is prerequisite for epidermal keratinization. TG3 that is also found in mammalian keratinocytes plays an important role in the formation of the stratum corneum in the skin by the introduction of cross-links into proteins. Hence, other functions of TG2/Gh protein such as a role in transmitting signals from classical G-coupled receptors might be involved in the transdifferentiation. Further study must be done to resolve the function of TG2/Gh in retinoid-pretreated skin. Because more information could be obtained in mammalian skin, we are now studying the TG2/Gh function using rat embryonic skin.

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