J Oral Pathol Med (2004) 33: 622–8
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Retinoic acid disintegrated desmosomes and hemidesmosomes in stratified oral keratinocytes

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BACKGROUND: Although it is known that retinoic acid (RA) regulates the cellular differentiation of skin keratinocytes, the effects of RA on the anchoring junction have not been clarified. The effects of all-*trans* RA on cell–cell and cell–matrix connections of gingival epithelial (GE)1 cells in a multilayered culture were investigated.

METHODS: Ultrastructures of GE1 cells were observed and immunohistochemistry was used to detect keratin 4, keratin 13, and desmoglein expression. Reverse transcription-polymerase chain reaction was performed to detect expression of desmosome and hemidesmosome-associating adhesion molecules, keratin 13, and keratin14.

RESULTS: Retinoic acid caused immunohistochemical diminution of keratin 4, keratin 13, and desmoglein. Ultrastructurally, RA induced drastic loss of typical desmosomes and complete loss of hemidesmosomes. RA significantly decreased the transcript levels of keratin 13, keratin 14, desmoglein 1, and desmocollin 1 in a dose-dependent manner. The 230-kD bullous pemphigoid antigen (BPAG1) gene expression was also reduced by RA, whereas transcript levels of integrin α6, integrin β4, the 180-kD bullous pemphigoid antigen (BPAG2), and laminin 5 were not affected.

CONCLUSION: These results indicated that RA disintegrated not only desmosomes by depriving the cells of desmoglein 1, desmocollin 1, keratin 13, and keratin 4, but also hemidesmosomes by reducing the expression of BPAG1 and keratin 14 in basal keratinocytes.

J Oral Pathol Med (2004) **33**: 622–8

Keywords: oral keratinocytes; retinoic acid; desmosome; hemidesmosome; bullous pemphigoid antigen; keratin; polymerase chain reaction

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Accepted for publication April 22, 2004

Introduction

Cell junctions occur at points of cell-cell and cell-matrix contact in all tissues, and they are particularly abundant in stratified squamous epithelia. Anchoring junctions mechanically attach cells to their neighbors or to the extracellular matrix and have two functionally different forms (1). One form includes adherens junctions and desmosomes, which hold cells together and are formed by transmembrane adhesion molecules that belong to the cadherin family. The other includes focal adherens and hemidesmosomes, which bind cells to the extracellular matrix and are formed by transmembrane adhesion molecules that belong to the integrin family. The transmembrane adhesion molecules of desmosomes are desmoglein and desmocollin, and those adhesion molecules of hemidesmosomes are integrin α6 and integrin β4 (2). Desmoglein and desmocollin connect to the intracellular cytoskeletal intermediate keratin filaments through anchor proteins such as plakoglobin and desmoplakin. On the contrary, integrin α6β4 connects the basal surface of an epithelial cell to the underlying basal lamina, and an intracellular domain of these molecules binds via an anchor protein (plectin) to keratin intermediate filaments as well as desmosomes (1). For the function of epithelia of skin and mucosa, the integrated structure of desmosomes and hemidesmosomes is undoubtly requisite. This is evident in hereditary skin blistering diseases such as junctional epidermolysis bullosa (EB) (3–6) and autoimmune diseases such as pemphigus (7–9) and pemphigoid (2).

The biological effects of retinoids on the epidermis are intimately linked to the control of proliferation and differentiation of keratinocytes. Therapeutic retinoid caused skin fragility through decreased numbers of tonofilaments in keratinocytes and desmosomal attachments in the epidermis of patients with disorders of keratinization (10). A retinoic acid (RA)-induced decrease in numbers of tonofilaments and desmosomes has also been reported in culture of embryonic chick skin (11) and in a human keratinocyte cell line (HaCaT cells) (12). Generally, the major effect of RA *in vitro* is thought to be suppression of keratinocyte differentiation,

as a result of reduction in a number of differentiation markers such as keratins 1 and 10 (13, 14). Notwithstanding, the regulating effect of RA on stratification of keratinocyte on the basis of cell-cell contact was insufficiently discussed, and the effect of RA on cell-matrix connection such as hemidesmosome was not known at all even in HaCaT cells. Therefore, we investigated the effect of RA on integration of cell junctions and the expression of adhesion molecules associating with desmosomes or hemidesmosomes in gingival epithelial (GE)1 cells.

Materials and methods

Cell culture of gingival epithelial cells

The GE1 cell line was established from GE tissue of temperature-sensitive SV40 large T-antigen gene transgenic C57BL/6 mice (15). Cells were cultured on plastic dishes in a chemically defined medium, SFM101 (Nissui, Tokyo, Japan), with 1% fetal bovine serum and 10 ng/ml EGF at the permitted temperature (33°C). Calcium concentration of the SFM101 medium was 1.13 mM. Cells grew exponentially, appeared in the confluent state at about day 10 of culture, and then formed a three-dimensional architecture of the stratified epithelium. Therefore, the cells were cultured for more than 10 days and further cultured with 0.1, 1 nM, 0.01, 0.1, 1 μM RA (Sigma Aldrich Japan, Tokyo) for more than 10 days for electron microscopic observation and 5 days for reverse transcription-polymerase chain reaction (RT-PCR). Ethanol was used for dissolving RA and added to the medium. A concentration of ethanol was $<0.1\%$.

Transmission electron microscopy of GE1 cells in the absence or presence of RA

Cells cultured on plastic dishes in the presence or absence of 1 μM RA were fixed in 2.5% glutaraldehyde and were post-fixed with 1% OsO_4. Then the samples were treated using a routine procedure and embedded in epoxy resin for transmission electron microscopic observation as previously described (15). Ultrathin sections were stained by uranyl acetate and lead citrate and observed under a transmission electron microscope (H-7100, Hitachi, Japan).

Immunohistochemistry of anchoring junctional molecules in GE1 Cells

Cells cultured on cover glasses were fixed with ethanol : acetic acid (99:1) or Bouin's fixative. After blocking endogenous peroxidase with methanol containing 0.3% hydrogen peroxide for 15 min, the sections were incubated with primary monoclonal antibodies against desmoglein (DG3.10, Progen Biotechnik, Heidelberg; Germany, 1:100), keratin 4 (6B10, Progen Biotechnik, 1:50), keratin 13 (M1C7, ICN Pharmaceuticals, Inc., OH, USA, 1:50), and keratin 14 (LL002, YLEM, Roma, Italy, 1:50) overnight at 4°C. Then they were further incubated with peroxidase-conjugated EnVision+™ reagent (Dako, CA, USA) for 30 min at room temperature. Next, 3, 3′-diaminobenzidine was applied as the final chromogen, and nuclei were counterstained with hematoxylin.

RT-PCR analysis of desmosome and hemidesmosome-associating molecules in GE1 cells

The stratified cells were treated with RA for 5 days. Total RNA was purified from the cells using an RNeasy minikit (Qiagen Com., Tokyo, Japan). RT-PCR was performed as described previously (15). Briefly, with Superscript II reverse transcriptase (GIBCO-BRL, NY, USA), complementary DNA (cDNA) was synthesized from 1 μg of total RNA in the presence of oligo $(dT)_{8-12}$ hexamer (Amersham Pharmacia Biotech, Buckinghamshire, England) in a 20-μl reaction volume at 37°C for 60 min. One microliter of cDNA solution was amplified with *Taq* polymerase (Takara, Tokyo, Japan) in a volume of 12.5 μl using each set of primers. Primers for desmoglein 1 (accession No: X74335), desmocollin 1 (accession No: NM_013504), integrin α6 (accession No: X69902), keratin 14 (accession No: BT007186), plectin (accession No: BC024074), the 230-kD bullous pemphigoid antigen (BPAG1) (accession No: AF396877), the 180-kD bullous pemphigoid antigen (BPAG2) (accession No: L08407), and laminin 5 β3 (accession No: NM_008484) were made on the basis of information in each database (Table 1). Primers for β4 subunit of integrin (16), keratin 13 (17), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (18) were made on the basis of information in reports. After degeneration at 95°C for 2 min, amplification of cDNA was performed with 20–33 sequential cycles at 95°C for 30 s and 55°C for 30 s and then one cycle at 72°C for 6 min. The PCR products were then analyzed by electrophoresis on 1.8% agarose gels containing 0.1 μl Gelstar (Cambrex Bio Science, Rockland Inc., ME, USA) per milliliter in 0.5x TBE buffer (50 mM Tris, 50 mM boric acid, 2 mM EDTA, pH 8.0).

For each pair of gene-specific primers, semi-logarithmic plots of the amplified DNA fragment intensity as a function of cycle numbers were used to determine the range of cycles over which linear amplification occurred. The number of PCR cycles was kept within this range. The amount of amplified DNA in agarose gels was measured by a densitometer (ATTO, AE-6920M-05, Tokyo, Japan). The ratios of the DNA amount amplified with each set of primers to the amount of DNA amplified with a set of primers for GAPDH were calculated. The value of the ratio in the control group was set at 100%, and the relative values in dose groups to the control group value were calculated and statistic analysis was performed by one way classification of analysis of variance (ANOVA).

Results

Effects of RA on ultrastructure of GE1 cells

The GE1 cells cultured for more than 10 days in the control medium exhibited a stratified epithelium of several layers (Fig. 1a). They had relatively large nuclei and prominent tonofibrils in the cytoplasm. These cells were connected to one another by many desmosomes (Fig. 1a, inserted figure), and the basal cells had adhered to the plastic dishes by hemidesmosomes (Fig. 2a, arrows). These hemidesmosomes showed intact

Table 1 Primers used for polymerase chain reaction

Genes	*Primer sequences*	*Position*	*Fragment size (bp)*	*Sequence references*
Desmoglein 1	5′-CACATTTTGGCACGACCACA-3′	218-237	101	X74335
	5′-CATTGCCATAGCCCCAGAGGATC-3′	319-278		
Desmocollin 1	5′-TCGCCATACTTCACCCAAACT-3′	1201-1221	133	NM_013504
	5′-AGGATTGTGTATACCGCCTTCG-3′	1334-1315		
Integrin α 6	5′-TGCTGCTCAGAATATCAAGCTCC-3′	3130-3152	145	X69902
	5′-ATAGAGCCAGCATCAGAATCCC-3′	3275-3254		
Integrin β 4	5′-CGCCGTCTGGTAAACATC-3′	3213-3230	259	Walker and Menko (1999) (16)
	5′-AGTAGCTTCACCTGCAACTC-3′	3472-3453		
Plectin	5′-GAGTCTGCAGTGCTCGACATCT-3′	2576-2597	121	BC024074
	5′-ATCCAGTACCGACAGAGAGGA-3′	2695-2674		
Keratin 13	5′-GCTGATAGTGGCTTTGGAGGTGGCTATGG-3′	211-239	302	Richard et al. (1995) (17)
	5′-TTGTAGTAGGGGCTGTAGTCCCGCTCAGG-3′	512-485		
Keratin 14	5′-CCCTACTTCAAGACCATTGAGGAC-3′	545-568	128	BT007186
	5′-TCATGCGCAGGTTCAACTCT-3′	692-673		
Laminin 5 β3	5′-AAATGTGACCAGTGTGCCCCT-3′	1528-1549	522	AY035783
	5′-TGATCTGCACAATCTTGCTCT-3′	2050-2030		
BPAG1	5′-ATGGAAAATAGAATGCTTGA-3′	6649-668	499	NM_008484
	5′-GTTTTAGTATCAGTCAGCAT-3′	7148-7127		
BPAG2	5′-CATACGGAGCGAAGAAAAACG-3′	1207-1227	132	L08407
	5′-TCCAGAATCAGGAACTTGCAGTC-3′	1339-1317		
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′	586-605	452	Ercolani et al. (1988) (18)
	5′-TCCACCACCCTGTTGCTGTA-3′	1037-1018		

BPAG1, 230-kD bullous pemphigoid antigen; BPAG2, 180-kD bullous pemphigoid antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

morphology and were composed of intracellular electron-dense plaques, anchoring tonofilaments, and subbasal dense plates. An amorphous lamina lucida-like space was observed at the interface between basal cells and the surface of the dish. However, a lamina densa of the basement membrane was not formed because connective tissue was absent in the culture system. After having reached confluence, GE1 cells were cultured in a medium containing 1 μM RA for an additional 13 days. As a result, the cell layer became thin (Fig. 1b) and the cells substantially lost tonofibrils in the cytoplasm and typical desmosomes in all layers (Fig. 1b). The numbers and sizes of desmosomes in the cells were counted on electron microscopic photographs magnified 2,000 or 25,000 times, respectively. For counting, only cells containing nuclei were selected and, in addition, cells of the uppermost layer and the basal layer were excluded. The results were presented in Table 2. The numbers of desmosomes in the presence of RA were less than those in the absence of RA in four independent experiments ($P < 0.01$, Student's *t*-test,). The mean size of desmosomes was shorter in the culture with RA than without RA, but was no significant difference. Another interesting finding is that cells cultured with RA completely lost hemidesmosomes (Fig. 2b). In the cells treated with RA, there were no intracellular electron-dense plaques, anchoring tonofilaments or subbasal dense plates.

Immunohistochemical findings of desmoglein and keratin peptides in cells cultured with or without RA

In immunohistochemical examination, the keratin 13-positive cells covered almost the entire surface of stratified cell layers by connecting with neighboring cells (Fig. 3a). Treatment with 1 μM RA resulted in a decrease in the number of keratin 13-positive cells (Fig. 3d). Keratin 4-positive cells were focally present (Fig. 3b). RA diminished the size of keratin 4-positive foci (Fig. 3e). Desmoglein was strongly positive at the intercellular bridges in the absence of RA (Fig. 3c, arrowheads). The desmoglein-positive cells were smaller and the number of them was less than that in the presence of RA (Fig. 3f).

Effects of RA on mRNA levels of anchoring junctional molecules as determined by RT-PCR

The transcript levels of desmoglein 1 ($P < 0.05$), desmocollin 1 ($P < 0.05$), and keratin 13 ($P < 0.01$), and keratin 14 ($P < 0.01$) in GE1 cells were dose-dependently decreased by RA treatment for 5 days (Figs 4 and 5), and one-way ANOVA showed a significant difference in the effect of RA. On the contrary, mRNA levels of α6 and β4 subunits of specific integrin for hemidesmosome were not changed by RA treatment. Therefore, the transcript levels of other molecules involving hemidesmosomes such as BPAG1, BPAG2, plectin, and laminin 5 were examined by RT-PCR (Fig. 5). The transcript level of BPAG1 was dose-dependently reduced in the cells cultured with RA ($P < 0.05$), whereas the transcript levels of BPAG2 and laminin 5 were not altered and that of plectin was increased ($P < 0.01$).

Discussion

Stratified squamous epithelia are characterized by the presence of numerous desmosomes and hemidesmosomes. The importance of desmosomes and hemidesmosomes for structure and function of the epidermis has been demonstrated in the autoimmune skin diseases, pemphigus (7–9) and pemphigoid (2). Affected individuals produce antibodies against their own proteins of desmosome or hemidesmosome. Desmosome is

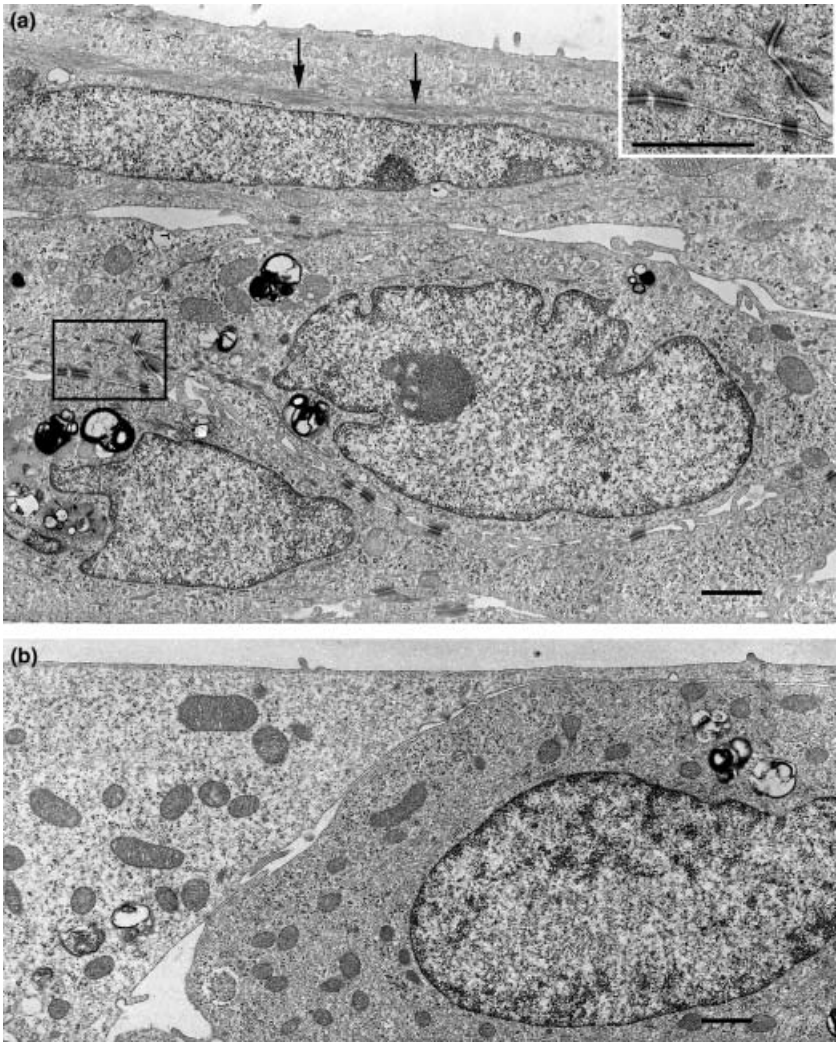

Figure 1 Transmission electron micrographs of GE1 cells in multilayered culture with or without RA. (a) GE1 cells cultured for 23 days in the control medium. Stratified keratinocytes include abundant tonofibrils (arrows) and are connected to one another by desmosomes (inserted figure). ×8,000, Bar = 1 μm. (b) GE1 cells cultured for 10 days in the control medium and consecutively cultured for an additional 13 days in the presence of 1 μM RA. This culture is composed of only two cell layers. Typical desmosomes are not seen at all. ×8,000, Bar = 1 μm.

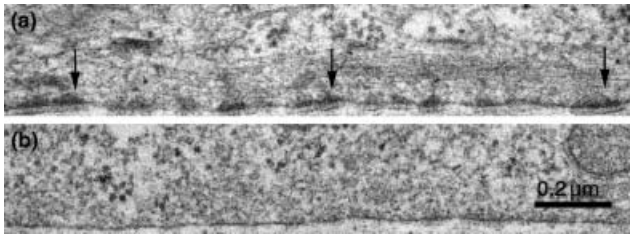

Figure 2 Transmission electron micrographs of GE1 cells cultured with or without RA. (a) Many hemidesmosomes (arrows) are seen in the plasma membrane facing the plastic dish in basal GE1 cells cultured in the control medium. ×48,000, (b) Hemidesmosomes are no longer seen in the plasma membrane of basal GE1 cells cultured with 1 μM RA for 13 days. ×48,000, Bar = 0.2 μm.

composed of transmembrane proteins such as desmoglein and desmocollin, which are connected to intracellular keratin intermediate filaments by mediating intracellular anchor proteins of desmoplakin and plakoglobin. Specific desmoglein and desmocollin isoforms are

Table 2 The number of desmosomes per cell and its size

	Control	*Treated with RA*	*Student's t-test*
Number of desmosomes per cell	6.2 ± 0.3 ($n = 4$)	2.6 ± 0.9 ($n = 3$)	$P < 0.01$
Size (μm)	0.254 ± 0.079 ($n = 33$)	0.169 ± 0.060 ($n = 19$)	NS

NS, not significant.

expressed at different stages of keratinocyte differentiation (7, 8, 19). Desmoglein 1 is restricted to the uppermost layers of the epidermis and desmoglein 3 is expressed in basal and spinous layers (7). In an *in vitro* study, RA caused shedding of HaCaT cells into the culture medium (12). Although HaCaT cells expressed desmoglein 2 and 3 and desmocollin 3, they expressed neither desmoglein 1 nor desmocollin 1. In HaCaT cells treated continuously with RA, loss of desmosomes

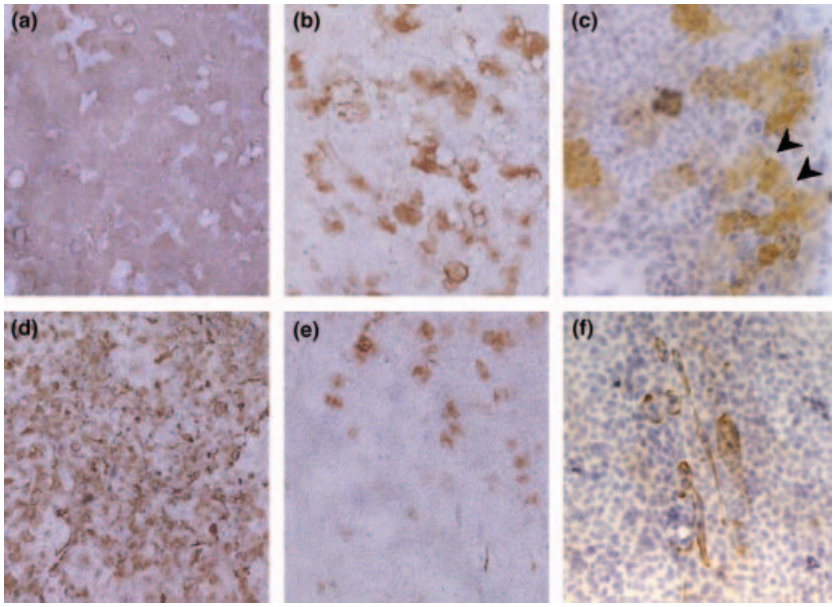

Figure 3 Immunohistochemistry of keratin 13 (a, d), keratin 4 (b, e), and desmoglein (c, f) in GE1 cells cultured with (d, e, f) or without RA (a, b, c). (a) In the control medium; keratin 13-positive flat cells covered almost all surface. (b) In the control medium; keratin 4-positive cells are focally present. (c) In the control medium; many flat desmoglein-positive cells connects each other. Arrowheads show desmoglein-positive intercellular bridges. (d) In the medium with RA; keratin 13-positive cells are smaller and cannot cover the surface. (e) In the medium with RA; keratin 4-positive foci are less and smaller than (b). (f) In the medium with RA. A few desmoglein-positive cells are present alone (a, b, d, e): ×30, (c, f): ×74.

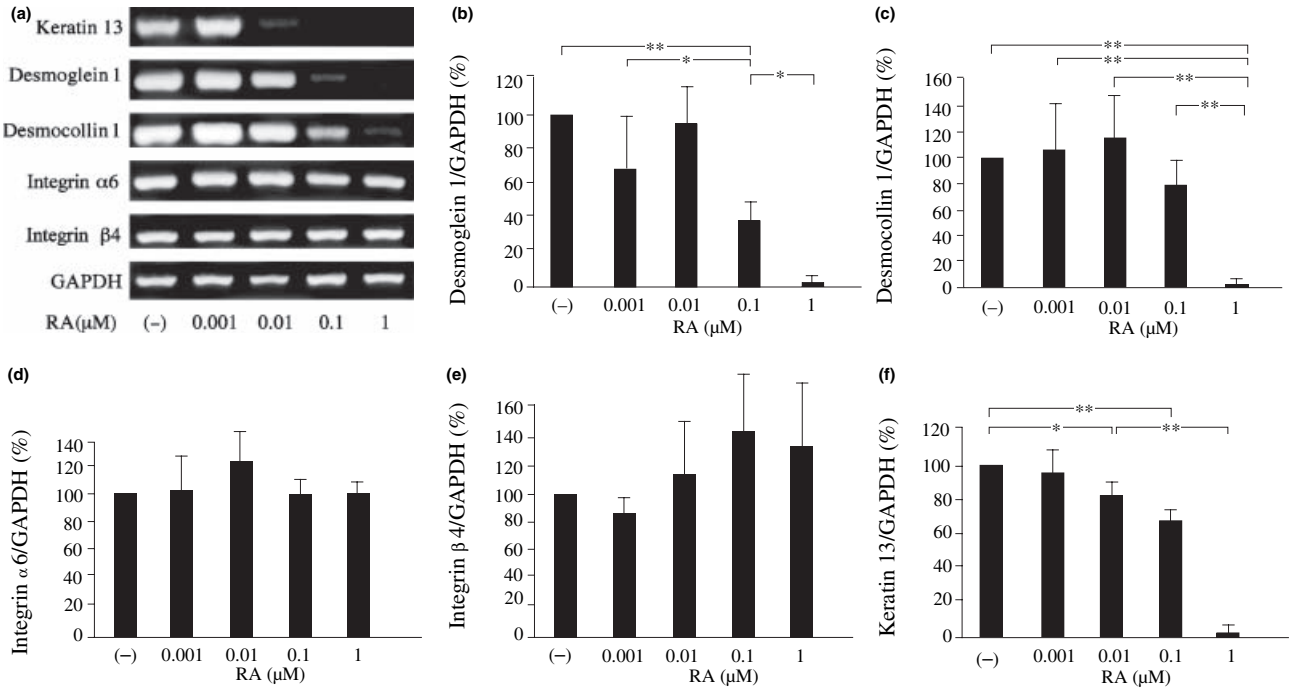

Figure 4 Effects of RA on mRNA expression of desmosome-relating molecules in GE1 cells. Cells were continuously treated for 5 days with RA at concentrations of 0.001, 0.01, 0.1, and 1 μM. RNA isolation, reverse transcription, and PCR were performed by the methods described in Materials and methods. (a) Agarose gel electrophoresis of amplification products of keratin 13, desmoglein 1, desmocollin 1, integrin α6, integrin β4, and GAPDH cDNA. (b–f) Relative values of desmoglein 1 (b), desmocollin 1 (c), integrin α6 (d), integrin β4 (e), and keratin 13 (f) to GAPDH. The results shown are the mean ± SD of three independent experiments. Significant difference from the culture without all-*trans* retinoic acid: $*P < 0.05$, $**P < 0.01$, ANOVA, Student's *t*-test.

occurred because of a reduction in the transcript levels of desmoglein 2, desmoglein 3, and desmocollin 3 (12). Unlike HaCaT cells, GE1 cells stratified in several layers and expressed desmoglein 1 and desmocollin 1, the levels of which were reduced by RA treatment following loss of desmosomes. These results indicated that the expression

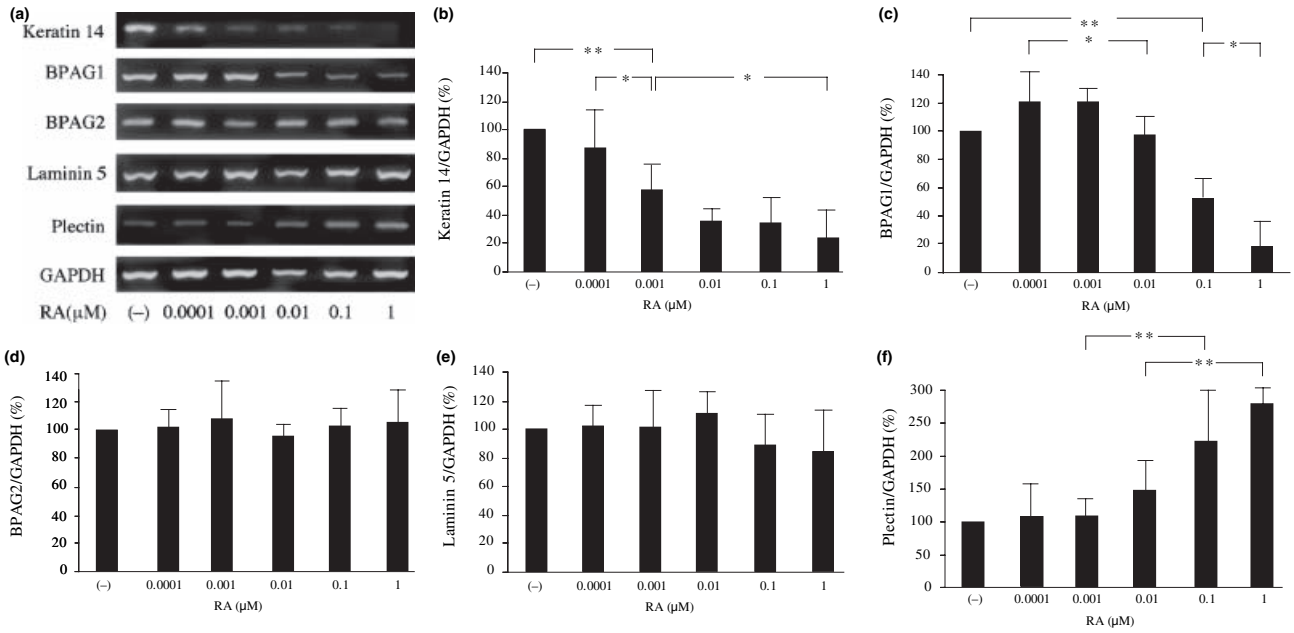

Figure 5 Effects of RA on mRNA expression of hemidesmosome-relating molecules in GE1 Cells. Cells were continuously treated for 5 days with RA at concentrations of 0.0001, 0.001, 0.01, 0.1, and 1 μM. RNA isolation, reverse transcription, and PCR were performed by the methods described in Materials and methods. (a) Agarose gel electrophoresis of amplification products of keratin 14, BPAG1, BPAG2, laminin 5, plectin, and GAPDH cDNA. (b–f) Relative values of keratin 14 (b), BPAG1 (c), BPAG2 (d), laminin 5 (e), and plectin (f) to GAPDH. The results shown are the mean ± SD of three independent experiments. Significant difference from the culture without all-*trans* retinoic acid: $*P < 0.05$, $**P < 0.01$, ANOVA, Student's *t*-test.

of desmoglein 1 and desmocollin 1 must be necessary for stratification composed of several cell layers, and therefore GE1 cells are more potential differentiated keratinocytes than HaCaT cells. Our results also showed that both desmoglein 1 and desmocollin 1 are critical proteins for maintenance of the integrated structure of desmosomes and stratification of keratinocytes.

Both of keratin 4 and keratin 13 are known to be common differentiation markers of stratified mucosal epithelia. Immuohistochemistry and RT-PCR showed that RA suppressed the expression of keratin 4 and keratin 13, and electron microscopic observation showed that tonofibrils had disappeared in the cytoplasm of RA-treated GE1 cells. As the keratin intermediate filaments are indirectly connected to one another through desmosomes (1), the reduction in the amount of keratin 4/13 was thought to be closely related to the disintegration of desmosomes.

Hemidesmosome consists of at least three distinct proteins (4): BPAG1, a component of an intracellular electron-dense plaque that serves as an autoantigen in bullous pemphigoid; BPAG2, a transmembrane collagenous protein that extends from the intracellular compartment to the extracellular space; and the basal keratinocyte-specific subunits of α6 and β4 of integrin (2). In addition, laminin 5 consists of anchoring filaments that traverse the lamina lucida (6). The best-understood group of hereditary skin blistering disorders, epidermolysis bullosa (EB), covers several inherited skin and mucosal fragility diseases. Different forms of EB are caused by defects in structural proteins comprising hemidesmosomes and the basement membrane zone (4). The relationship between inherited affected molecules and their abnormal electron microscopic features has been elucidated (3–6, 20–22). A subtype of junctional EB (Herlitz-type) ultrastructurally showed a lack of hemidesmosomes, for which laminin 5 was responsible. In contrast, we firstly showed that RA treatment-induced complete loss of hemidesmosome in GE1 cells. At this time, the gene expression of only BPAG1 among hemidesmosome-associating molecules was inhibited and the transcript level of laminin 5 was not altered. BPAG1 contributes to the formation of intracellular electron-dense plaques of hemidesmosomes (4). In GE1 cells treated with RA, the intracellular dense hemidesmosomal plaques disappeared completely. Therefore, it was suggested that the disintegration of hemidesmosomes was probably caused by a reduction in BPAG1. In addition, the decreased transcript level of keratin 14 induced loss of tonofibrils connected to the intracellular dense plaque via plectin. The marked reduction in the amount of keratin intermediate filaments was probably an additive cause of disintegration of hemidesmosomes through disruption of the cytoskeletal keratin network.

In conclusion, RA disintegrated not only desmosomes by depriving the cells of desmoglein 1, desmocollin 1, keratin 13, and keratin 4, but also hemidesmosomes by reducing the expression of BPAG1 and keratin 14 in basal keratinocytes.

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Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research (C) No. 15591944, High Performance Biomedical Materials Research (1998–2003), and Open Research Project (2000–2003) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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