

Original

Expression of connexins and the effect of retinoic acid in oral keratinocytes

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Abstract: Differential expression of members of the connexin (Cx) gap junction multigene family permits formation of gap junctions with the varied physiological properties required by different tissues. The aim of this study was to characterize connexin expression and the influence of all-*trans*-retinoic acid (RA) in mouse gingival epithelial cells (GE1). The cells were treated with RA, and expression of Cxs was analyzed by immunofluorescence, reverse transcriptase-polymerase chain reaction (RT-PCR), and real-time PCR. RT-PCR revealed that GE1 cells expressed mRNA for Cx26, Cx30.3, Cx31.1, Cx32, and Cx43. In addition, real-time PCR revealed that RA significantly decreased expression of Cx31.1 as compared with control. These results indicate that GE1 cells are useful in analyzing the expression of connexin molecules in oral keratinocytes from oral mucosal lesions. (J Oral Sci 53, 327-332, 2011)

Keywords: connexin; retinoic acid; oral keratinocytes; gingival epithelium.

Introduction

Gap junctions are specialized channels that form between two adjacent cells. They allow ions and mole-

cules less than 1 kDa to pass between the cells, thereby coupling the cells both electrically and metabolically (1,2). Connexins (Cxs) are transmembrane proteins that form gap junctions. Connexin proteins display characteristic differences in their sensitivity to voltage, changes in pH, phosphorylation, and response to pharmacological reagents, and these differences permit the formation of gap junctions with varied physiological properties. It is generally believed that gap junction-mediated intercellular communication is important for proper growth (3,4), differentiation (5-7), and function of cells and that the absence of intercellular communication is often associated with a transformed or cancerous phenotype (2,8).

Molecular studies have identified a large family of connexin genes encoding gap junction channel proteins in rodents (8,9) and humans (2,10). In human epidermis, connexin genes encode different proteins that participate in terminal differentiation (2). In recent years, a number of connexin genes have been implicated in hereditary skin diseases (2,11). Cx26 and Cx43 were identified in the oral mucosal epithelium (7,12-14); other subtypes of connexin have not yet been examined. The physiological properties of gap junctions and the functional mechanisms involved in the control of gap junction communication differ by connexin subtype. Thus, identification of connexin subtypes in oral mucosal epithelium is required if we are to better understand the causes of oral mucosal diseases and thereby determine the therapeutic effect of agents that might modify gap junctional intercellular communication.

Retinoic acid is believed to be an important factor in regulating the differentiation and function of epidermal cells. Indeed, all-*trans*-retinoic acid (RA) was found to

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Table 1 Primers used for reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time PCR

Genes	Primer sequences	Size (bp)	Sequence references	
<i>RT-PCR</i>				
Cx 26	Forward	5'-CGGAAGTTCATGAAGGGAGAGAT-3'	365	
	Reverse	5'-GGTCTTTTGGACTTTCCTGAGCA-3'		
Cx30.3	Forward	5'-GGTCTTTTGGACTTTCCTGAGCA-3'	103	
	Reverse	5'-AGGTCATGGATAACACCTGCA-3'		
Cx32	Forward	5'-AATGCACGTAGCTCACCAACAG-3'	101	
	Reverse	5'-TGCACCTGTGTCTCTTTACCTCT-3'		
Cx43	Forward	5'-GGCTGCTCCTCACCAACGGCC-3'	332	
	Reverse	5'-AGGTCATCAGGCCGAGGTCTG-3'		
GAPDH	Forward	5'-ACCACAGTCCATGCCATCAC-3'	452	Ercolani et al. (18)
	Reverse	5'-TCCACCACCCTGTTGCTGTA-3'		
<i>RT-PCR, Real-time PCR</i>				
Cx31.1	Forward	5'-GCTCGAGAGAAGAAGTACCAGGAA-3'	101	
	Reverse	5'-AGGCTGAAGACGTATGTCCACC-3'		
<i>Real-time PCR</i>				
Cx32	Forward	5'-GTCCCTGCAGCTTATCTTGG-3'	195	
	Reverse	5'-CGGAACACCACACTGATGAC-3'		

Abbreviations: Cx, connexin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

alter the expression of molecules responsible for cell-cell adhesion, such as desmosomes and tight junctions (15,16). In the present study, we aimed to determine the expression of gap junction-constituting molecules in oral mucosal keratinocytes and the effect of RA on their expression by immunofluorescence, reverse transcriptase-polymerase chain reaction (RT-PCR), and real-time PCR.

Materials and Methods

Cell culture of oral keratinocytes

An immortalized mouse gingival epithelial cell line (GE1) (17) was used. Cells were cultured on plastic dishes in a chemically defined medium, SFM 101 (Nissui, Tokyo, Japan), with 1% fetal bovine serum, 10 ng/ml EGF (Sigma Aldrich Japan, Tokyo, Japan), and 0.2% Antibiotic Antimycotic Solution ($\times 100$; Sigma Aldrich Japan) at the optimal temperature (33°C). Fetal bovine serum was removed from the medium during the experiment. After seeding of 2×10^5 cells in a 60-mm dish, the cells grew exponentially and were became confluent approximately day 10 of culture.

Immunofluorescence

Cells were cultured for 5 days on an eight-well chamber slide (BD, Tokyo, Japan) and were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 30 min. The cells were permeated with 0.1% Triton X-100 in PBS for 5 min, after which they were incubated

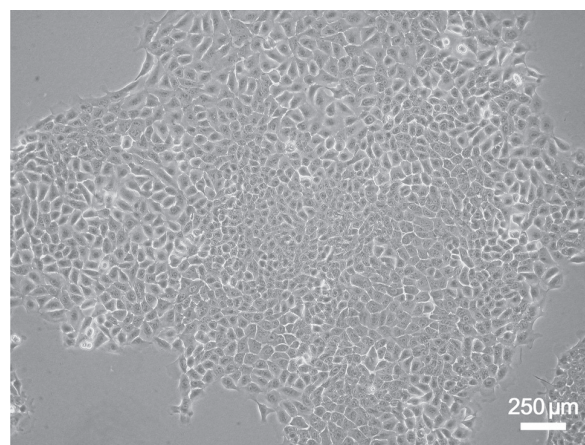


Fig. 1 Phase-contrast microscopic view of GE1 cells at day 5 of culture. Stratification of cell layers is not yet evident.

for 2 h at 37°C with each primary antibody against Cx26 (1:100, Zymed Laboratories Inc., South San Francisco, CA, USA), Cx31.1 (1:100, Santa Cruz Biotechnology, Inc., San Francisco, CA, USA), Cx32 (1: 100, Santa Cruz Biotechnology, Inc.), and Cx43 (1: 100, Santa Cruz Biotechnology, Inc.). After washing with PBS containing 0.5% Tween 20, cells were incubated with Alexa Fluor® 488- or 546-conjugated secondary antibodies (1:200, Molecular Probes Inc., Eugene, OR, USA) for 1.5 h at 37°C and were counterstained with 0.1 $\mu\text{g/ml}$

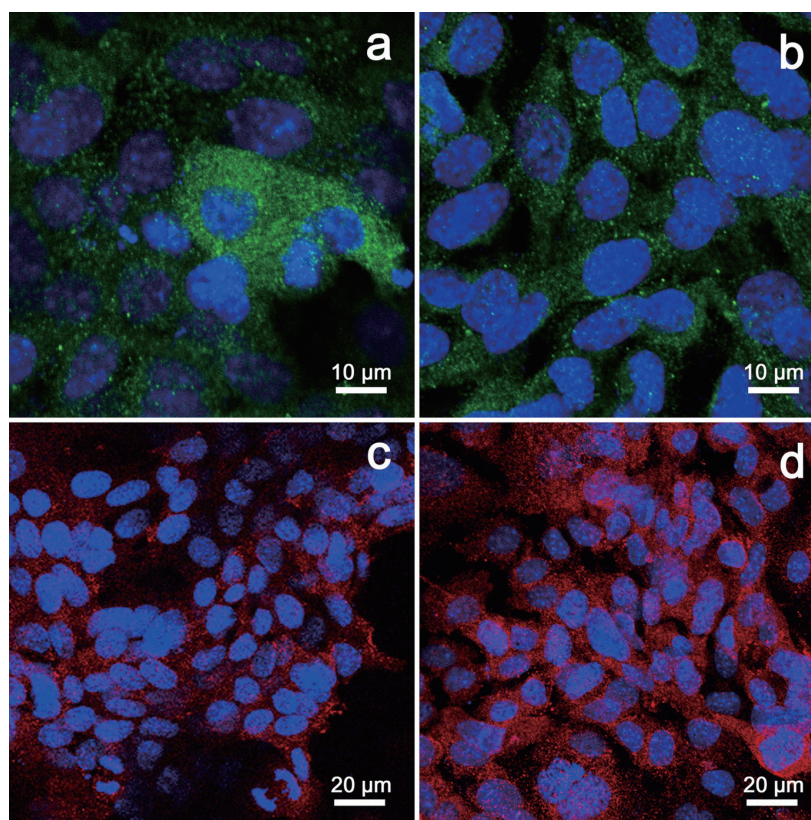


Fig. 2 Immunofluorescence of Cx26 (a), Cx31.1 (b), Cx32 (c), and Cx43 (d) in GE1 cells. Cx26 is stained in the cytoplasm in upper cells (a, green), Cx31.1 is stained in the cytoplasm of almost all cells (b, green), Cx32 is stained in the cytoplasm (c, red), and Cx43 is strongly stained at the intercellular border (d, red).

4'-diamidino-2-phenylindole (DAPI, Molecular Probes, Inc.) for 20 min. Then the sections were inspected with a C1 spectral imaging system (Nikon Instrument Com., Tokyo, Japan). The negative control was incubated with non-immune serum instead of the primary antibodies.

RT-PCR

On the fourth day after the initial cell seeding, the medium was replaced with medium containing 1 nM, 0.01 μ M, 0.1 μ M or 1 μ M RA (Sigma Aldrich Japan). RA was dissolved with ethyl alcohol; in the control group, ethyl alcohol at a concentration of 0.1% was added to the medium. The cells were continuously cultured for an additional 5 days.

Total RNA was collected from almost confluent cells at 9 days after seeding with an RNeasy minikit (Qiagen Com., Tokyo, Japan). With SuperScript reverse transcriptase (GIBCO-BRL, NY, USA), complementary DNA was synthesized from 1 μ g of total RNA in the presence of oligo (dT)₈₋₁₂ hexamer (Amersham Pharmacia Biotech, Buckinghamshire, UK) in a reaction volume of 20 μ l

at 37°C for 60 min. One microliter of cDNA solution was amplified with *Ex Taq* polymerase (Takara, Tokyo, Japan) in a volume of 12.5 μ l, using each set of 20- μ M primers. The primer sequences used for PCR are shown in Table 1. The PCR products were then analyzed by electrophoresis on 1.8% agarose gels containing 0.01% Gelstar (Cambrex Bio Science, Rockland Inc., ME, USA) in 0.5x TBE buffer.

Real-time PCR

The samples that showed a dose response for RA were analyzed by real-time PCR using the 7500 Fast Real-Time PCR System (Applied Biosystems, Tokyo, Japan) for quantitative evaluation. One microliter of cDNA solution was amplified with Power SYBR Green PCR Master Mix (Applied Biosystems) in a volume of 25 μ l using each set of 10- μ M primers. The primer sequences for Cx31.1 were identical in RT-PCR and real-time PCR; the sequences used for Cx32 are shown in Table 1. One-way analysis of variance (ANOVA) followed by Dunnett's test were used for statistical analysis.

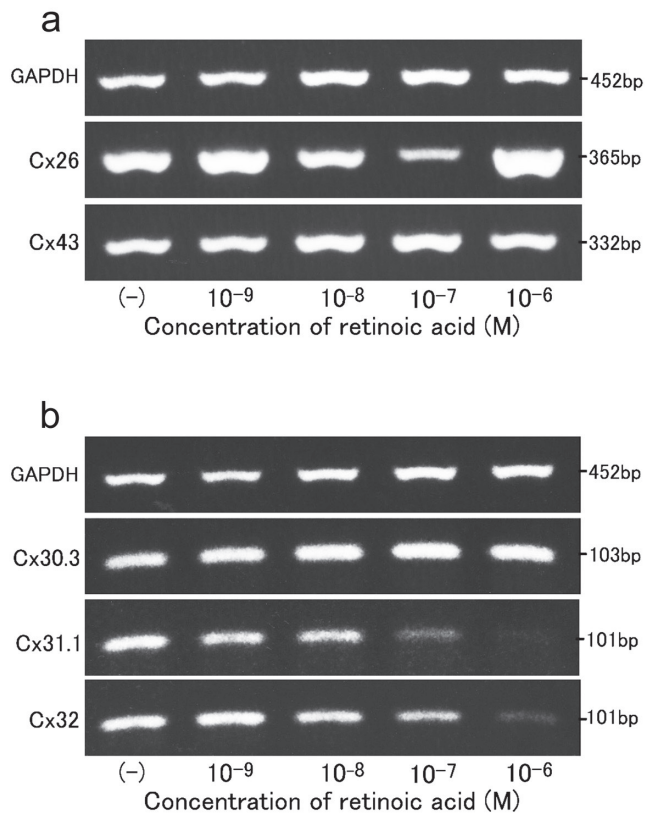


Fig. 3 Representative agarose gel electrophoresis of the RT-PCR amplification products of connexins in GE1 cells. Cells in (a) and (b) were cultured in medium with RA for 5 days, and total RNA was purified. RT-PCR was then performed and separated by electrophoresis.

Results

Immunofluorescence of Cx26, Cx31.1, Cx32, and Cx43

GE1 cells proliferated and had a cobblestone appearance (Fig. 1). Cells cultured for 5 days did not reach 100% confluence. The presence of Cx26, Cx31.1, Cx32, and Cx43 in GE1 cells was examined by immunofluorescence. Cx26 was clearly present in the cytoplasm of upper cells (Fig. 2a). Cx31.1 was also present in the cytoplasm of all cells (Fig. 2b). The intensity of staining of Cx32 was weak (Fig. 2c), whereas staining of Cx43 was strong and intercellular borders were immunostained (Fig. 2d).

mRNA expression of connexins on RT-PCR and real-time PCR

The expressions of five different connexin family members in GE1 cells were analyzed by RT-PCR. Mouse-specific connexin primers revealed that GE1 cells express Cx26 (Fig. 3a), Cx30.3 (Fig. 3b), Cx31.1 (Fig.

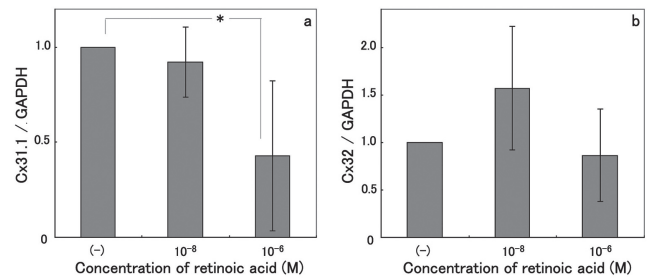


Fig. 4 Relative values of Cx31.1 (a) and Cx32 (b) to glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The results shown are the mean \pm SD of three independent experiments. There was a significant difference in Cx31.1 (a) but not Cx32 (b) between cells cultured with and without retinoic acid (ANOVA). * $P < 0.05$

3b), Cx32 (Fig. 3b), and Cx43 (Fig. 3a) mRNA. Of these, Cx31.1 and Cx32 mRNA were diminished by RA treatment for 5 days. Subsequent real-time PCR revealed a dose-dependent reduction in Cx31.1 ($P < 0.05$) with RA concentration (Fig. 4). Cx32 mRNA expression was not reduced in the group treated with 10^{-8} M RA.

Discussion

To understand the contribution of a particular connexin to gap junction function, it is necessary to characterize the expression and distribution of connexin molecules within a given tissue and cell type. The epidermis is primarily composed of keratinocytes, which temporally and spatially express as many as 10 different connexins, depending on their degree of differentiation (2,6). In addition, the importance of gap junctions in epidermal differentiation is supported by the discovery that mutations in gap junction proteins are the underlying cause of several inherited skin diseases (2,11). In rat epidermis, Cx26, Cx31, Cx31.1, Cx37, and Cx43 were demonstrated by immunofluorescence and Northern blot analysis (8), and Cx26, Cx30, Cx30.3, Cx31, Cx31.1, Cx32, Cx37, Cx40, Cx43, and Cx45 mRNAs have been detected in human epidermis (10), although Cx32 expression was low. Expression of Cx26, Cx31, Cx40, Cx43, and Cx45 was found in mouse skin (9), but expression of Cx32 was not examined. We were unable to identify any other studies of Cx32 expression in skin in our review of the literature on connexin expression. In our study, RT-PCR confirmed keratinocyte expression of Cx26, Cx31, and Cx43, as well as expression of Cx30.3, Cx31.1, and Cx32, in mouse oral mucosa. Taken together, these findings suggest that substantial expression of Cx32 occurs

in mucosal keratinocytes, but not in epidermis.

Retinoic acid is an effective modulator of proliferation and differentiation of keratinocytes *in vivo* and *in vitro*. It was reported that although increased expression of Cx43 occurred at low (10^{-11} M) concentrations, reduced expression was observed at high (10^{-7} M) concentrations in epidermal keratinocytes, even though gap junctional communication, as measured by fluorescent dye transfer, was not altered (19). An increase in Cx43 expression and induction of Cx26 expression in human epidermis by RA treatment were also reported (20). In contrast, there was no change in the expression of Cx43 or Cx26 in oral keratinocytes in the present study, whereas the expression of Cx31.1 mRNA decreased in response to RA treatment. A previous study demonstrated that expression of Cx31.1 was more abundant in mature rat skin than in fetal skin (8) and that Cx31.1 protein was localized in differentiated layers, including the upper spinous and granular layers (2). These studies indicate that differentiated keratinocytes express Cx31.1. Our previous study showed that RA inhibited terminal differentiation of oral keratinocytes by triggering the disappearance of desmosomes in RA-treated cells (15). These findings suggest that reduced expression of Cx31.1 in RA-treated cells is due to inhibition by RA of terminal differentiation in oral keratinocytes. To investigate the possibility of RA-induced changes in the expression of other connexin subtypes, it will be necessary to accumulate data on their localization and to analyze the association with the terminal differentiation of oral keratinocytes.

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