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Retinoic Acid Regulates Oral Epithelial Differentiation by Two Mechanisms

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The effect of retinoic acid (RA) concentration on differentiation of oral keratinocytes and the influence of fibroblasts on RA-dependent regulation were investigated in a lifted culture system. Keratinocyte differentiation was assessed by morphology, immunohistochemistry and immunoblotting. Filaggrin/profilaggrin and keratin 1 were used as biochemical markers for cornified epithelium and keratins 13 and 19 as markers for noncornified epithelium. Cultured oral keratinocytes in RA-free conditions differentiated in a manner that closely resembled the differentiation pattern of gingival epithelia in vivo. Increasing RA concentrations altered the in vivo-like terminal differentiation of oral keratinocytes by disruption of organized stratification, inhibition of filaggrin/profilaggrin and K1 expression, and stimulation of K13 and K19 expression. Differentiation of keratinocytes from both cornified and noncornified regions of the oral cavity varied in a similar manner in response to added RA, with the exception of K19 expression. K19 was consistently expressed at higher levels in keratinocytes originating from noncornified epithelia as compared to those from cornified epithelia. The level of RA regulation was ultimately dependent on the type of fibroblasts underlying the epithelial cells. Homologous fibroblasts rendered the oral keratinocytes less sensitive to the effects of RA than skin fibroblasts. In addition, at a given RA concentration, fibroblasts from cornified oral mucosa potentiated keratinocyte expression of RA sensitive markers of keratinization as compared to the influence exerted by fibroblasts originating from noncornified oral mucosa. These results indicate that the RA regulation of oral epithelial differentiation is mediated by two separate mechanisms: a direct, RA concentration-dependent effect, and an indirect, fibroblast-mediated effect. Key words: oral mucosa/vitamin A/dermal equivalent. J Invest Dermatol 104:546–553, 1995

The oral cavity exhibits extensive regional variation in epithelial differentiation. This characteristic makes oral epithelia an excellent model for studies of differentiation processes in epithelia. The two major differentiation patterns of oral epithelium, the noncornified epithelium of lining mucosa and the cornified epithelium of masticatory mucosa, can be distinguished by expression of a number of structural proteins [1–3]. For example, the noncornified epithelia express keratin 13 and 4 in suprabasal cell layers and keratin 19 in basal cells, whereas the cornified epithelium expresses only small amounts of K13 and the major keratin products in suprabasal cells are keratins 1 and 10 along with keratins 6 and 16. Profilaggrin, a keratin-associated protein of epidermis, is also expressed in oral cornified epithelia [4].

Vitamin A exerts profound effects on differentiation of epithelial tissues; hypervitaminosis causes mucous metaplasia, whereas deficiency leads to squamous metaplasia ([5], review). The response of epidermal keratinocytes to retinoic acid (RA), the active form of vitamin A in epithelia [6], has been shown by numerous studies [7–10]. In epidermal keratinocytes cultured at the air-liquid interface, which undergo a high degree of differentiation, RA inhibits morphologic structure and expression of several markers of cornified epithelia, such as K1 and profilaggrin [11,12]. In contrast, keratins 13 and 19, normally found in noncornified epithelia, are upregulated by RA in these experimental conditions. These observations suggest a possible role of RA in region-specific differentiation of oral epithelia.

RA affects gene transcription via interaction with specific nuclear receptor proteins that function as retinoid-activated transcription factors [13–15]. To date, three different retinoic acid receptor subtypes (RARα, RARβ, and RARγ) have been cloned and sequenced. Strong interspecies sequence homology suggests that each RAR subtype has distinct cellular functions ([15,16], review). It has recently been shown that the transcription of a number of differentiation markers in human epidermal keratinocytes is controlled by RA/RAR complexes [17,18]. In addition, a second family of retinoid responsive nuclear receptors (RXRs), which preferentially bind the RA metabolite 9-cis-RA, has been characterized [19]. RXRs form heterodimers with RARs and function as co-regulators of gene transcription [20]. Another set of proteins thought to be involved in mediating RA actions in cells are the cellular retinoic acid-binding proteins I and II (CRABP-I and CRABP-II). CRABP-II is the predominant form in human keratinocytes [21] and has been postulated to maintain appropriate
Table I. Antibodies Used for Identification of Markers of Epithelial Differentiation

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<thead>
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<th>Antibody</th>
<th>Marker for</th>
<th>Source</th>
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<tbody>
<tr>
<td>SC10</td>
<td>Keratin K1</td>
<td>T.-T. Sun*</td>
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<tr>
<td>AE8</td>
<td>Keratin K13</td>
<td>A. Schermer and T.-T. Sun*</td>
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<tr>
<td>KS19.1</td>
<td>Keratin K19</td>
<td>Unpublished results ICN, Immunobiologicals, Lisle, IL</td>
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<tr>
<td>AKH-I</td>
<td>Profilaggrin/flaggrin</td>
<td>Date et al, 1987 [48]</td>
</tr>
<tr>
<td>8959 (blots)</td>
<td>Profilaggrin/flaggrin</td>
<td>Fleckman et al, 1985 [49]</td>
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* These antibodies were generously provided by T.-T. Sun.

intracellular RA concentrations for differential regulation of gene transcription [22,23].

Connective tissue is also important in determining epithelial differentiation [24]. Studies by recombination of oral and dermal tissues have shown that the connective tissues are able to influence differentiation and proliferation of keratinocytes [25-27]. However, the molecular mechanisms of epithelial-mesenchymal interactions in the oral cavity are still largely unknown and the role of retinoids is not understood. Sangster et al. [28] showed that dermal fibroblasts modulate the effects of retinoids on epidermal growth. It is therefore conceivable that subepithelial fibroblasts may also modulate the retinoid effect on adult skin and oral keratinocyte differentiation.

The aim of this study was to achieve in vivo-like differentiation of oral keratinocytes in culture to investigate the effects of RA and the influence of subepithelial fibroblasts in oral keratinocyte differentiation in vitro. This work is a first step in understanding a possible role of retinoids in region-specific differentiation of oral epithelia. Our results demonstrate two levels of the RA effect on differentiation of oral keratinocytes: a direct RA concentration-dependent regulation, which is similar for keratinocytes from both cornified and noncornified oral mucosa, and an indirect regulation mediated by the underlying fibroblast type. Both mechanisms are likely to be involved in the region-specific differentiation of oral epithelia.

MATERIALS AND METHODS

Cell Culture

Oral tissues were obtained from patients undergoing routine oral surgical procedures. Epithelial cells were separated from the underlying tissue by dispase treatment [29], and primary cultures were grown on plastic in keratinocyte growth medium (KGM) (Clonetics Co., San Diego, CA). Keratinocytes were expanded through one passage in KGM.

Lifted cultures were grown essentially as described by Asselineau and Pruinieres [30]. Briefly, bovine type I collagen (Bioeica, France) was mixed with a suspension based on minimum essential medium (Gibco, Life Technologies, Inc., Grand Island, NY), 10% fetal bovine serum (FBS) (Hyclone Laboratories Inc., Logan, UT) and 2 × 10^6 fibroblasts per plate. Fibroblasts were tested included GM10 fibroblasts, a cell line from human fetal dermal tissue (N.I.G.M.S., Camden, NJ), and oral and dermal fibroblasts, obtained from appropriate tissues according to conventional explant technique [31]. The collagen lattices were allowed to contract for 37°C for 3-7 d, depending on fibroblast type. Second-passage keratinocytes originating from either cornified or noncornified oral mucosa were then seeded on the contracted collagen lattices at a density of 2 × 10^4 cells/cm². The cultures were grown submersed in Dulbecco's modified Eagle's medium (Gibco) for 1 week, then raised to the air-liquid interface and grown for two more weeks. The medium contained 10% of either normal (control) or delipidized (FBS). Delipidization was carried out according to the method of Rothfalt et al. [32] and the delipidized product was redissolved at an equivalent protein concentration in Hepes buffered saline. During the first week, all culture media were supplemented with normal FBS, penicillin/streptomycin (Gibco), hydrocortisone (0.04 µg/ml) (Sigma), choroethoxin (10⁻⁶ mol/l) (Calbiochem Behring Diagnostics, La Jolla, CA), putrescine (5 µg/ml) (Sigma). At the time of plate elevation, all media, except the control medium, were switched to delipidized FBS and 1000X stock solutions of all-trans RA in isopropyl alcohol (Sigma) were added together with the above supplements under yellow light to a final RA concentration of 0, 10⁻⁸, 10⁻⁷, 10⁻⁶, or 10⁻⁵ mol/l. Cultures were kept in the dark and media changed twice per week. Retinoid concentration in control serum was 5 × 10⁻⁷ mol/l retinol as determined by the Clinical Nutrition Research Laboratory at the University of Washington using the technique of Bieri et al. [33]. Retinol was not detectable in the delipidized serum by this technique.

Epithelial Differentiation Analysis

Two lattices were used for each experimental group. One specimen was fixed in methyl Carnoy's, sectioned, and used for light microscopy of hematoxylin and eosin-stained sections and for immunohistochemical analysis by the avidin biotin peroxidase complex method [34]. The other lattice was used for protein analysis using 7.5-12.5% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblottting. The cultured epithelium was mechanically separated from the collagen lattice and epithelial proteins were extracted by homogenization in Urea-Tris buffer (8 mol/l and 0.05 mol/l, pH 7.6), as described previously [35]. The extracts were frozen at −70°C until analysis. Discontinuous sodium dodecyl sulfate gels were performed by the technique of Laemmli [36] and stained with Coomassie brilliant blue. Proteins from duplicate gels were electrophotorectively transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH) and protein markers of interest were identified with appropriate antibodies (Table I) employing the avidin biotin peroxidase method.

RESULTS

Oral Culture Parameters

The dermal equivalent culture system [30] was modified as described above to allow for in vivo-like oral keratinocyte differentiation. A comparison of morphology between oral cornified epithelium and cultured oral keratinocytes grown in medium containing 10% delipidized serum at the air-liquid interface is shown in Fig 1. The cultured epithelium was more compact and lacked the prominent epithelial ridges seen in the gingival tissue. However, the cultured epithelium did exhibit the four characteristic layers of a normal gingival epithelium, i.e., basal, spinous, granular, and cornified cell layers. A partially dissociated zone of cells was seen above the well differentiated cell layers in most cultures. These cells were most likely formed early in the culture period prior to exposure to differentiating conditions at the air-liquid interface and remained associated with the differen-
Figure 2. RA inhibits terminal differentiation of gingival keratinocytes in lifted cultures—immunohistochemistry. Morphology (hematoxylin and eosin) and expression of profilagrin (AKH-1), keratin 1 (5C10), keratin 13 (AE8), and keratin 19 (KS19.1) by gingival keratinocytes is regulated by RA. Cells were grown at the air-liquid interface on GM-10 fibroblast containing lattices in media with delipidized serum and with various RA concentrations added back as indicated. Interrupted lines mark the epithelial border. Bar, 100 μm.

tiated culture due to lack of desquamation under these conditions [37].

Immunohistologic staining for four representative differentiation markers is also shown in Fig 1. In the tissue as well as in the cultured epithelium, the granular layer was well delineated by reaction with antibody AKH-1, showing a band of profilagrin expression immediately below the cornified cell layer. Both epithelia exhibited suprabasal expression of K1 as detected by antibody 5C10. In contrast, K13 was expressed only in occasional, individual cells in the tissue and was also very poorly expressed in the differentiating portion of the cultured epithelium. K19 was not expressed in either epithelium.
Differentiation of oral keratinocytes is extremely sensitive to culture conditions. Epithelial cells of higher passage number (greater than three) or those that grew slowly in secondary cultures were less well differentiated when transferred to lattices (data not shown), suggesting the necessity of using standardized conditions for growth and expansion of these cells. Second-passage keratinocytes (tertiary) expanded for approximately two and a half weeks were used in subsequent experiments.

Effect of Retinoic Acid on Oral Epithelial Differentiation

The effect of RA on cornified oral keratinocytes in dermal equivalent cultures is shown in Fig. 2. In medium containing no RA or low concentrations of RA (10^{-6} mol/l) a squamous stratified epithelium was formed and markers of cornified differentiation, profilaggrin and K1, were expressed. High-RA conditions (10^{-8} mol/l) prevented terminal differentiation of the keratinocytes, resulting in a disorganized epithelium with increased expression of markers of noncornified epithelium, K13, and K19, and decreased expression of markers of cornified epithelium, profilaggrin, and K1. The immunoblot in Fig 3 further support the findings of the immunohistochemistry. An increase in K19 expression (KS19.1) was observed with increasing RA concentrations, and a decrease in profilaggrin/filaggrin and K1 expression (8959 and 5C10) occurred under these conditions.

Keratinocytes from oral noncornified mucosa responded similarly to RA in a parallel series of experiments (data not shown). To control for inter-experimental variation, keratinocytes from cornified and noncornified oral mucosa taken from one patient were compared for RA response in a single experiment. Differentiation of these two cell types in response to RA was similar (Fig 4). In RA-free conditions, both cell types formed a prakeratinized, stratified epithelium with similar expression of profilaggrin, K1, and K13. In 10^{-8} mol/l RA, both keratinocyte types formed an epithelium that was less well keratinized, but again morphology and expression of profilaggrin, K1, and K13 were comparable. In contrast, expression of K19 differed consistently between these two epithelia in various growth conditions (Fig 4 and data not shown). K19 expression was consistently higher in the cultured epithelia originating from noncornified oral mucosa, mimicking the pattern of expression seen in the original tissues [38].

Influence of Fibroblasts on Oral Epithelial Differentiation

The above results indicated that although there was some degree of intrinsic diversity (K19 expression) between the basal cells of cornified and noncornified oral epithelium, this diversity was not sufficient to explain the variation in regional differentiation in situ. This suggested that extrinsic signals influence the pathway of terminal keratinocyte differentiation. One source of extrinsic signals is the subepithelial fibroblast population. Therefore, we tested the ability of different fibroblast types to influence the RA-sensitive differentiation of oral keratinocytes.

Fibroblasts were introduced as the variable in three separate comparisons of oral keratinocyte differentiation. In medium containing 10% control serum (comparable to approximately 10^{-8} mol/l RA), oral keratinocytes influenced by fetal dermal fibroblasts (GM108) expressed markers of noncornified oral epithelia, K13, and K19, and, conversely, when influenced by oral fibroblasts (HOFs 91-14) expressed markers of cornified epithelia, profilaggrin, and K1 (Fig 5). Results at three different RA concentrations (0, 10^{-7}, and 10^{-7} mol/l, data not shown) support this finding and suggest that in comparison with oral fibroblasts, human fetal fibroblasts potentiated the RA response of the keratinocytes. When adult oral fibroblasts (HOFs 91-14) and adult dermal fibroblasts (J.G.) were compared, a similar effect was observed. The epithelial cells cultured with dermal fibroblasts differentiated as if they were exposed to higher amounts of retinoid than when cultured with oral fibroblasts. This difference was most prominent at high RA concentrations (Fig 6), where 10^{-7} mol/l RA resulted in disruption of orderly stratification in the cultures with dermal fibroblasts (J.G.), but not with oral fibroblasts (HOFs 91-14).

The final comparison was done between fibroblasts originating from oral cornified mucosa (HOFs 92-21) and oral noncornified mucosa (HOFs 92-22). Of these two oral fibroblast subtypes, those from cornified epithelia (HOFs 92-21) induced a lower RA sensitivity in the keratinocytes, promoting expression of markers of cornification, profilaggrin, and K1, and inhibiting markers of noncornified epithelia, K13, and K19 (Fig 7). The differences in RA sensitivity can also be seen in the morphology of these cultured epithelia (Fig 8). This difference was most prominent in the high RA concentration, as the effect of seemingly higher RA concentration (compare to Fig 6) emerged in the cultures containing fibroblasts from noncornified oral epithelia (HOFs 92-22).

Although the epithelial cells displayed differential RA sensitivity when influenced by fibroblasts from either cornified or noncornified oral mucosa, the difference was not sufficient to implicate subepithelial fibroblasts as the sole regulators of regional variation in oral epithelial differentiation.

DISCUSSION

The aim of this work was to investigate RA effects on oral epithelial differentiation. An air-liquid interface culture system was adapted for growth and differentiation of oral keratinocytes. This culture system was used to show that RA inhibits morphologic differentiation and expression of biochemical markers of keratinization of oral epithelial cells irrespective of the original in situ keratinocyte differentiation. In addition, we showed that subepithelial fibroblasts play an important role in the regulation of the RA responsiveness of oral keratinocytes and subsequent differentiation.

The observation that RA acts as an inhibitor of oral cornified keratinocyte differentiation parallels the findings of Asselineau et al [12], who demonstrated a similar RA effect on epidermal differentiation. They showed that a specific range of RA concentrations (10^{-9} - 10^{-8} mol/l) was required to obtain in vitro epithelial architecture closest to that found in vivo. A lower optimal range was found in the present study. In fact the oral keratinocytes seemed to differentiate equally well in delipidized media with no added RA as in delipidized media with low RA concentrations (10^{-10} mol/l).

This difference in RA levels for optimal differentiation between the two studies may be due to differences in culture conditions or to inherent differences between epidermal and oral keratinocytes. In the present study, we have found inherent differences between two...
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<tr>
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<td>Noncornified</td>
<td>Cornified</td>
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<td><img src="image9.png" alt="Image" /></td>
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<td>AE8</td>
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<td>KS19.1</td>
<td><img src="image17.png" alt="Image" /></td>
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Figure 4. Keratinocytes from cornified and noncornified oral epithelia differentiate similarly in response to RA in lifted cultures. Keratinocytes from the same donor from the two major differentiation patterns of oral epithelium were grown at the air-liquid interface on lattices containing gingival fibroblasts. The growth media were supplemented with delipidized serum either without RA or with RA added back to a final concentration of $10^{-8}$ mol/l. Markers of differentiation were analyzed by immunohistochemistry (same as in Fig 2). Note the similar expression of markers in the two epithelial types, except for K19 (KS19.1), which was more strongly expressed in noncornified epithelia under all conditions tested. Also note that in the high RA concentrations, the use of oral fibroblasts here yields more satisfactory morphology and differentiation than with GM10 fibroblasts used in Fig 2. Interrupted lines mark the epithelial border. Bar, 100 μm.

oral keratinocyte types in their keratin 19 expression. Evidence of inherent differences between various keratinocyte populations is also presented by Lindberg and Rheinwald [39], who identified three distinct subtypes of human oral keratinocytes based on differentiation patterns in xenografts subsequent to primary cultures.

Our results, however, indicate that regional variation in oral epithelial differentiation cannot be entirely explained by intrinsic differences in the two keratinocyte types and their response to RA. We suggest an important role of the subepithelial fibroblast. The fibroblasts were able to influence differentiation of the oral keratinocytes in accordance with their tissue of origin via an apparent modulation of keratinocyte RA sensitivity. Consequently, fibroblasts from cornified oral mucosa inhibited the RA response of oral keratinocytes, thus leading to more abundant expression of markers of cornification in the epithelial cells, whereas fibroblasts from
noncornified epithelia potentiated the RA response of oral keratinocytes.

Some previous studies suggest that the adult oral epithelial phenotype is an intrinsic property of the epithelium [39–41]. Other observations indicate that connective tissues are responsible for the final pattern of oral epithelial differentiation [42–44]. The present study suggests that neither the intrinsically predetermined keratinocyte phenotype (see Fig 4), nor the influence of regional fibroblasts (see Fig 8) are by themselves capable of inducing the large variation in terminal differentiation seen in the oral cavity. Consequently, this suggests that there must be a continuous cross talk between the oral keratinocytes and the oral fibroblasts during the induction process leading to terminal differentiation of the epithelial cells. Furthermore, we have shown in the present study that evaluation of the epithelial response to signals from the underlying connective tissues may depend on the differentiation marker measured. Thus, the adult oral keratinocyte phenotype may be intrinsically predetermined in some regards (K19 expression), but receptive for extrinsic influences in others (K1, K13, and profilaggrin expression).

![Figure 5. Human fetal dermal fibroblasts (GM10) induce greater RA sensitivity in gingival keratinocytes than gingival fibroblasts (HOFs 91-14). Gingival keratinocytes were grown on lattices containing either GM10 fibroblasts or HOFs 91-14 in medium with 10% control serum. These conditions approximate 10^{-8} mol/l RA (see Materials and Methods). Markers of differentiation were assessed by immunohistochemistry (same as in Fig 2). Note prominent expression of K13 (AE8) and K19 (KS19.1) in epithelia cultured with GM10 fibroblasts, but expression of K1 (5C10) and filaggrin (AKH-1) in cultures with oral fibroblasts. Interrupted lines mark the epithelial border. Bar, 100 μm.](image1)

![Figure 6. Adult dermal fibroblasts (J.G.) and gingival fibroblasts (HOFs 91-14) induce different responses in RA-sensitive differentiation of gingival keratinocytes. Epithelial morphology of gingival keratinocytes grown on lattices containing either J.G. fibroblasts (dermal) or HOFs 91-14 (gingival) in medium with 10^{-8} mol/l RA was assessed by hematoxylin and eosin staining. Note that gingival keratinocytes form a noncornified type epithelium in the presence of gingival fibroblasts, but a disorganized epithelium is formed in the presence of dermal fibroblasts at this RA concentration. Bar, 100 μm.](image2)

![Figure 7. Fibroblasts from different regions of oral cavity differentially influence expression of RA-sensitive markers of keratinocyte differentiation—immunoblots. Keratinocytes were cultured on lattices containing either fibroblasts from cornified (C) or noncornified (N) oral mucosa in media with delipidized serum supplemented with 10^{-7} mol/l or 10^{-9} mol/l RA. Proteins were separated on a 7.5% to 12.5% gradient sodium dodecylsulfate gel, transferred to nitrocellulose, and probed with antibodies as indicated (KS19.1 is specific for keratin 19, AE8 for keratin 13, 5C10 for keratin 1, and 8959 detects profilaggrin and filaggrin). The first panel shows a duplicate gel stained with Coomassie brilliant blue. Molecular weight markers (kDa) are indicated on the left and proteins of interest on the right.](image3)
Figure 8. Fibroblasts from different regions of oral cavity differentially affect morphology of gingival keratinocytes grown at the air-liquid interface. Keratinocytes were cultured on latissimus containing fibroblasts from either cornified (C) or noncornified (N) oral mucosa in media with delipidized serum supplemented with $10^{-7}$ mol/L or $10^{-9}$ mol/L RA and morphology was assessed by hematoxylin and eosin staining. Note the greater sensitivity of keratinocytes to toxic effects of RA, i.e., disruption of orderly stratification, when cultured in the presence of fibroblasts from a noncornified region. Bar, 100 μm.

Certain variability between consecutive experiments in the present study can be attributed to the use of primary cultures for each experiment. However, this variation does not alter the interpretation and conclusions of the results reported. The effects of RA on oral keratinocyte differentiation and the influences of the various fibroblasts were consistently observed. Furthermore, to ensure reliability, most of the results shown in this report were derived from comparisons within a single experiment and any direct comparison between experiments was made with caution.

This study indicates that the dermal equivalent is a useful model system for studies of oral keratinocyte differentiation. It will complement the mouse-xenograft model [39] as a system for study of highly differentiated oral keratinocytes. One advantage of the dermal equivalent is the ease of administering various agents influencing oral epithelial differentiation. Our results suggest that at least two different RA-associated regulatory mechanisms influence terminal differentiation of oral epithelia: one is via a direct, RA-concentration-dependent mechanism. In this regulation low RA concentrations decreased expression of the two markers of noncornified oral epithelia, K13 and K19, whereas expression of the two markers of cornification, profilaggrin and K1, were increased and high RA concentrations had the opposite effect. Keratinocytes from cornified and noncornified oral regions showed no difference in the sensitivity for this type of RA regulation for three of the four markers examined in the present study. Only K19 expression was consistently different in these two keratinocyte types at various RA concentrations. This phenomenon may be explained by differential expression of RAR subtypes in the two epithelial cell types. Thus, for example, RARγ, which is thought to be equally expressed in the two cell types, may control K13, K1, and profilaggrin expression, whereas RARβ, which is differentially expressed in these two cell types, may control K19 expression [45,46].

We show here that a second level of regulation, affecting RA-sensitive markers of differentiation, is mediated by subepithelial fibroblasts. The nature of this regulation is more indirect, because it seems to influence the apparent RA exposure of the keratinocytes independently of the actual RA concentration in the culture medium. One possible mechanism for this kind of regulation may be exerted via the CRABPs through their proposed ability to sequester RA in the cytoplasm of epithelial cells [21], preventing it from acting as a ligand for the nuclear RARs. This effect may in turn be mediated by tissue-specific expression of one or more soluble fibroblast proteins that influence epithelial cells. Indeed, Boukamp et al have demonstrated that demins regulates epidermal keratinocyte growth and differentiation via diffusible factors [47]. The dermal equivalent systems will serve as a basis for future experiments aimed at clarification of these hypotheses.

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