**Effect of Retinoids on Hyperproliferation-Associated Keratins K6 and K16 in Cultured Human Keratinocytes: A Quantitative Analysis**

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The keratin patterns of human epidermal keratinocytes cultured on a 3T3-feeder layer in the presence of $10^{-8}$ M non-aromatic (all-trans retinoic acid and 13-cis retinoic acid) and polyaromatic (arotinoid, arotinoid-sulfone, and free arotinoid-acid) retinoids were analyzed by high resolution one- and two-dimensional gel electrophoresis and immunoblotting. Laser densitometric evaluation of one-dimensional gels allowed to quantitate the changes within the keratin patterns and revealed an increase in the expression of keratins K13, K15, and K19 as induced by both non-aromatic and polyaromatic retinoids, except for the parent compound arotinoid. This would then indicate that such keratinocytes are pursuing a more embryonic type of differentiation.

In evaluating the data for the hyperproliferation-associated keratins K6 and K16 we noticed an unexpected result: except for all-trans retinoic acid, these two keratins showed opposite responses. As compared to control cultures, the amount of K6 did generally increase, while K16 was reduced, with arotinoid acid being the most effective retinoid. The apparently uncoupled expression of K6 and K16 appeared also to be concentration dependent when 13-cis retinoic acid at concentrations of $10^{-9}$, $10^{-8}$, and $10^{-7}$ M were analyzed.

Considering the overall antiproliferative potency of retinoids, we therefore conclude that K16 alone, rather than the pair K6/K16, should be regarded as a proliferation-related keratin and as such may be used as a sensitive marker to evaluate keratinocyte proliferation. *J Invest Dermatol* 95:450–455, 1990

Keratins comprise a heterogenous family of acidic (pI 4.5–5.5) and neutral-to-basic (pI 6.5–7.5) proteins ranging in apparent molecular weight from 40,000 to 67,000. They represent the major structural components of the water-insoluble intermediate filament system in epithelial tissues [1–6].

Keratins are usually expressed as specific pairs of acidic (type I) and neutral-to-basic (type II) subunits, both of which are essential for filament formation [7–12]. For example, K5 and K14 are characteristically produced in the basal layer of stratified squamous epithelia [13,14], whereas, in the course of stratification and differentiation, more complex patterns of keratin expression are manifested: terminally differentiating epidermal cells express K1 and K10 [2,5,6], suprabasal corneal cells express K3 and K12 [14,15,16], and stratified layers of the upper digestive tract epithelium express K4 and K13 [17,18].

In response to epidermal trauma, such as during wound healing but also in psoriatic lesions, the suprabasal cells down-regulate their expression of K1 and K10 and induce the expression of K6 and K16, which are therefore called hyperproliferation-associated keratins [19–26]. Although the induction of K6 and K16 correlates with an increase in proliferation of epidermal cells, it has not yet clearly shown that this switch is indeed functionally important. Vitamin A, and its derivatives, are essential for normal epithelial differentiation [27,28]. There are several reports indicating retinoids to influence keratin gene expression in a number of different ways. Non-aromatic retinoids, at high concentrations, are able to suppress the expression of K1 and K10 in cultured epidermal keratinocytes and to induce expression of K13 and K19, instead [29–33]. Considering the successful therapy of psoriasis and other dermatological disorders by retinoids [34], the molecular pathways by which these compounds exert their effects still need to be unraveled.

In this report, we describe the influence of non-aromatic [all-trans retinoic acid (all-trans RA), 13-cis retinoic acid (13-cis RA)] and polyaromatic retinoids (arotinoid, arotinoid sulfone, free arotinoid acid) on keratin expression of cultured human epidermal keratinocytes using quantitative laser densitometry of extracted keratins separated by high resolution one- and two-dimensional gel electrophoresis. We have observed an induction of K13, K15, and K19 and a reduced expression of the hyperproliferation-associated keratin K16, while, except for all-trans RA, K6 was unaltered or slightly increased. We therefore conclude that K16 alone may serve as a sensitive indicator of keratinocyte proliferation.

**MATERIALS AND METHODS**

**Epidermal Cell Cultures** Human keratinocytes were isolated from split skin of the thigh by incubating the specimens in PBS (pH 7.2) containing 0.25% trypsin for 2 h, followed by mechanical agitation. Keratinocytes were seeded at a density of $1 \times 10^4$ cells/cm² and grown on a 3T3-feeder layer [35,36] in modified Dulbecco's...
modified Eagle's medium supplemented with 10% fetal calf serum, 10 ng/ml epidermal growth factor, 10^{-8} M cholera toxin, 0.4 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.5 μg/ml amphotericin B. The medium was changed every third day.

For the investigations, keratinocytes in their first subculture were used. On day 2, medium supplemented with non-romatic (all-trans RA, 13-cis RA) polyaromatic (arotinoid, -sulfone, -acid) retinoids at the concentration of 10^{-8} M was added and changed every other day for 10 d. 0.2% DMSO served as control vehicle. Human keratinocytes were also cultured under the above conditions with all-trans RA and 13-cis RA at concentrations of 10^{-9} and 10^{-7} M.

**Preparation of Keratin-Enriched Protein Fractions**

The 3T3 feeder layer was selectively removed from confluent cultures of epidermal keratinocytes with 0.02% EDTA in PBS (pH, 7.2) as described [37]. The remaining keratinocytes, from three parallel cultures, were then harvested into a centrifuge tube, homogenized by ultrasonic disruption in 20 mM Tris/HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride and 5 μg/ml each of peptatin and antipain, and extracted twice with 600 mM KCl, 5 mM ethylenedinitrilotetraacetic acid (EDTA), 5 mM ethylene bis(oxyethylene)nitrilo)tetraacetic acid (EGTA), 50 mM Tris/HCl, pH 7.4, 1% Triton X-100, and the protease inhibitors as described above.

Each extraction step included ultrasonic disintegration and centrifugation at 27,000 × g. The resulting pellet, highly enriched in cytoskeletal proteins, was solubilized in sample buffer (0.5 M Tris/HCl, pH 6.8, 2.1% SDS, 10% mercaptoethanol, 10% glycerol, and bromphenolblue) and heated for 10 min at 90°C.

**Gel Electrophoresis and Densitometry**

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [38] and two-dimensional analyses by non-equilibrium pH gradient electrophoresis (NEpHGE) according to O'Farrell et al [39] were performed as described by Mischke and Wild [40]. Gels were stained in 0.2% Serva-Blue R. For quantitation of protein bands separated by NEpHGE/SDS-PAGE, a LKB Ultra-Scan XL laser densitometer connected with an IBM-PC PS 2/50 was used. K5 (K5b or K5a respectively [17,40]) served as an internal reference for equal protein loading and for quantification, because keratin K5 apparently is unaffected by retinoids both at the protein and the mRNA level [30,32]. Thus, the measured levels of the other keratins represent their ratio with respect to K5. The quantitation was performed with the soft-ware program “GelScan XL” (version 1.64).

**Immunoblot Analysis**

Proteins from unstained one- and two-dimensional gels were transferred by semi-dry blotting onto nitrocellulose sheets as described by Towbin et al [41]. Unspecific protein binding sites were blocked in 0.14 M TBS (10 mM Tris/HCl, pH 7.4, and 140 mM NaCl) supplemented with 0.05% Tween 20 and 15% horse serum. The filters were incubated overnight with antikeratin antibodies, washed twice with 0.14 M TBS/0.05% Tween 20, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit (BIO-RAD, München, FRG) as secondary antibody. Monoclonal mouse antikeratin antibodies were bridged via rabbit anti-mouse IgGs (Dakopatts, Denmark). After washing twice with 0.14 M TBS/0.05% Tween 20 and 3 times with 0.14 M TBS, the peroxidase reaction was developed using 0.02% 4-chloro-1-naphthol (freshly prepared as a 0.3% solution in methanol) and 0.015% hydrogen peroxide in 0.14 M TBS.

The anti-keratin antibodies used were the commercially available monoclonal antibodies C_{4} 4.62 recognizing K19 and K13, and C_{5} 8.12 recognizing K16 and K15 (both from ICN, Eschwege), and the rabbit polyclonal antisera 8-2/4 and 10-2/2 discriminating the acidic (type I) and the neutral-to-basic (type II) keratin subfamilies, respectively ([40], Wild G and Mischke D, unpublished).

**RESULTS**

Homogenization and extraction of cultured human keratinocytes with high salt/Triton X-100 containing buffer yielded water-insoluble cytoskeletal residues highly enriched in keratin proteins.

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**Figure 1.** Two-dimensional separation by NEpHGE/SDS-PAGE of human keratins from epidermal keratinocytes cultured without additional retinoids (control). (a) Coomassie Blue stained gel, (b) immunoblot, reaction with the monoclonal antibodies C_{4} 4.62 (K19) and C_{5} 8.12 (K13, K16), (c) immunoblot, reaction with 8-2/4 (predominantly type I keratins), (d) diagram of keratins expressed in cultured human epidermal keratinocytes (on 3T3 feeder layer). Keratins are numbered according to the catalog of keratins [4] considering the polymorphic keratins [40]. Keratin pairs are shown with identical symbols.

**Figure 2.** Two-dimensional separation by NEpHGE/SDS-PAGE of keratins from human epidermal keratinocytes growing in the presence of arotinoid acid (10^{-8} M). (a) Coomassie Blue stained gel, (b) immunoblot, reaction with the monoclonal antibody C_{4} 4.62, (c) immunoblot, additional reaction with C_{5} 8.12, (d) immunoblot, additional reaction with 8-2/4.
Although the quantitative evaluation of K17 revealed a variable response of this keratin, K14 showed opposing effects with respect to the two groups of retinoid tested. Non-aromatic retinoids led to a reduction and polyaromatic retinoids to an increase of the amount of keratin synthesized for this keratin (Table I), thus indicating an altered expression of K14 with respect to its usually coexpressed type II partner keratin K5.

When evaluating the data for the hyperproliferation-associated keratins K6 and K16, we observed an unexpected result: except for all-trans RA, the expression of K6 increased, whereas K16 was down-regulated. Again, arotinoid acid was the most effective compound (Table I).

In order to investigate this apparently uncoupled expression of K6 and K16 more thoroughly, we have compared keratin expression in human epidermal keratinocytes cultured under the influence of all-trans RA and 13-cis RA at concentrations of 10⁻⁹, 10⁻⁸, and 10⁻⁷ M (Fig 3). At 10⁻⁹ M, both retinoids induced K6 and K16. In contrast, at the higher concentrations all-trans RA inhibited the expression of both K6 and K16, whereas 13-cis RA had no or only a slightly increasing effect on the expression of K6, but, again, led to a reduction in K16 expression (Fig 4, Table II).

In addition, a concentration-dependent increase of K13, K15, and K19 expression was found that was more pronounced for all-trans RA than for 13-cis RA (Table II).

**DISCUSSION**

The biochemical, immunologic, and laser densitometric analyses of the keratin profiles of human cultured keratinocytes confirm the considerable potency of non-aromatic and polyaromatic retinoids to influence the growth and differentiation of keratinocytes as reflected in the regulation of keratin expression.

**Induction of K13, K15, and K19 Expression by Retinoids**

Although K13, K15, and K19 are usually expressed separately in many adult epithelia, their combined expression occurs in embryonic development of epidermis. For example, K13 is transiently expressed in the two- and three-layered epithelia of fetuses [46, 47], whereas K16 is down-regulated. Again, arotinoid acid was the most effective compound (Table I).

**Interference of Retinoids with K6/K16 Expression and Proliferation**

Several studies have described the abnoromral expression of K6 and K16 in diseases of the epidermis [19-26, 52] concluding effective retinoids. Although the increase of K13 brought about by arotinoid acid was uncompetitive due to insufficient protein loading, it became apparent on two-dimensional gels (Fig 2b, c, compare to Fig 1b). For the parent compound, arotinoid, no effect was detectable.

10⁻⁸ M, alterations within the keratin patterns were apparent. In order to quantitate these changes, keratin-enriched fractions separated by one-dimensional gel electrophoresis were identified by immunoblot analysis were evaluated by laser densitometry. To facilitate direct comparisons among the samples, we have used K5 as an internal reference, because this keratin has been shown to be unaffected by retinoids both at the protein and the mRNA level [30, 32]. Thus, the amount of keratin determined by laser densitometry is given as its ratio with respect to K5.

As compared to control cultures, the measurements then revealed an appreciable increase in expression of K13, K15, and K19 (Table I). In this respect, all-trans RA and arotinoid acid were the most

**Table I. Effect of Non-aromatic and Polyaromatic Retinoids (10⁻⁸ M) on Keratin Expression**

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>K6</th>
<th>K16</th>
<th>K13</th>
<th>K15</th>
<th>K19</th>
<th>K14</th>
<th>K17</th>
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<tr>
<td>All-trans RA</td>
<td>-9</td>
<td>-3</td>
<td>+10</td>
<td>+31</td>
<td>+79</td>
<td>-18</td>
<td>+6</td>
</tr>
<tr>
<td>13-cis RA</td>
<td>+6</td>
<td>-2</td>
<td>+5</td>
<td>+20</td>
<td>+4</td>
<td>-29</td>
<td>-11</td>
</tr>
<tr>
<td>Arotinoid</td>
<td>+7</td>
<td>-3</td>
<td>n.d.</td>
<td>+1</td>
<td>-20</td>
<td>+1</td>
<td>+39</td>
</tr>
<tr>
<td>Arotinoid sulfone</td>
<td>+11</td>
<td>-2</td>
<td>n.d.</td>
<td>18</td>
<td>+20</td>
<td>+21</td>
<td>+39</td>
</tr>
<tr>
<td>Arotinoid acid</td>
<td>+31</td>
<td>-34</td>
<td>n.d.</td>
<td>+33</td>
<td>+60</td>
<td>+28</td>
<td>+12</td>
</tr>
</tbody>
</table>

* Laser densitometric evaluation of keratin synthesized in cultures of human epidermal keratinocytes in the presence of 10⁻⁸ M retinoids. Values indicate changes (absorption unit ^* measured as compared to DMSO (control) cultures.

^* mm, not detectable.

Figure 3. One-dimensional SDS-PAGE of human keratins expressed in cultured human epidermal keratinocytes. (a) Coomassie Blue stained gel, (b) corresponding immunoblot reacted with a mixture of the rabbit polyclonal antisera 8-2/4 and 10-2/2 to detect all keratins. Lane 1, human epidermis; lane 2, DMSO (control); lane 3, all-trans RA, 10-7 M; lane 4, all-trans RA, 10-8 M; lane 5, all-trans RA, 10-9 M; lane 6, 13-cis RA, 10-7 M; lane 7, 13-cis RA, 10-8 M; lane 8, 13-cis RA acid, 10-9 M. Open arrowheads, type I; closed arrowheads, type II keratins.

The biochemical, immunologic, and laser densitometric analyses of keratin profiles of human cultured keratinocytes confirm the considerable potency of non-aromatic and polyaromatic retinoids to influence the growth and differentiation of keratinocytes as reflected in the regulation of keratin expression.
that K6/K16 expression seems to be associated with an increase in epidermal proliferation. Therefore, it has been suggested that this pair of keratins be used as biochemical markers for hyperproliferating keratinocytes [15,24].

In the present study we have evaluated the expression of K6 and K16 in cultured human keratinocytes following treatment with a set of different retinoids. In agreement with other reports [53,54], all-trans RA did inhibit the expression of both K6 and K16 in a concentration-dependent manner. For all other retinoids tested, however, the paired expression of K6 and K16 became uncoupled, with retinoic acid being the most effective compound. The marked inhibition of K16 expression correlates well with the results measuring 3H-thymidine incorporation into DNA by autoradiography, when keratinocytes are cultured in the presence of polyaromatic retinoids [55]. Therefore, a close correlation between proliferation and expression of K16 in normal cultured keratinocytes must exist.

However, a recent report [53] emphasizes that expression of K6/K16 and epidermal hyperproliferation may not always be linked, because a marked inhibition of K6/K16 expression, but no decline in cell proliferation, was observed in the human squamous cell carcinoma line SCC-13, when cells were cultured on floating rafts at the air-liquid interface and exposed to retinoic acid.

The reason for the different findings in normal epidermal keratinocytes and in SCC-13 cells may be related to the specific retinoid used, because we also detected a weak suppression of proliferation and K6/K16 expression by all-trans RA, whereas all-trans RA, generally accepted to have a higher antiproliferative capacity, revealed the marked inhibition of just K16. Finally, it should be considered that SCC-13 is a tumor cell line derived from a facial epidermal tumor that had received series of radiation treatments before surgical removal [56]. Hence, we would be rather cautious to compare normal and neoplastic proliferation behavior, the more as it is not completely understood how neoplastic growth is initiated and maintained.

Thus, considering both proliferation and K16 gene expression being decreased under the influence of retinoids, K16 apparently is the only proliferation-associated keratin. Support for this conclusion may also be drawn from the particular distribution of K16 in hyperproliferative epidermis as assessed by monoclonal antibody studies [57].

Knowing about the antiproliferative effects of retinoids on keratinocytes in cell culture and in therapy of psoriasis [34,58,59] we therefore suggest that K16 may be used as a sensitive marker to evaluate the antiproliferative potency of retinoids in non-transformed keratinocytes. The question, however, whether modulation of K6/K16 expression is a cause or a frequent consequence of the hyperproliferative state remains and more experiments will be necessary to further define the molecular pathways by which retinoids exert their action on proliferation and on regulation of keratin expression.

Reflecting clinical application of all-trans RA and 13-cis RA, the observed uncoupled expression of K6/K16 may explain why oral application of 13-cis RA is much better tolerated than oral treatment with all-trans RA, which is impossible because of its side effects. Thus it is conceivable that the linked suppression of K6 and K16 results in a non-tolerable intervention of homeostasis of the cell whereas the uncoupling of K6 and K16 expression just exerts an imbalance within the intermediate filament system which, possibly, can be better compensated.

### Table II. Concentration-Dependent Changes in Keratin Expression

<table>
<thead>
<tr>
<th></th>
<th>K6</th>
<th>K16</th>
<th>K13</th>
<th>K15</th>
<th>K19</th>
<th>K14</th>
<th>K17</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-trans RA, 10-8 M</td>
<td>+12</td>
<td>+4</td>
<td>+2</td>
<td>+17</td>
<td>+8</td>
<td>-23</td>
<td>-3</td>
</tr>
<tr>
<td>All-trans RA, 10-9 M</td>
<td>-9</td>
<td>-3</td>
<td>+10</td>
<td>+31</td>
<td>+79</td>
<td>-18</td>
<td>+6</td>
</tr>
<tr>
<td>All-trans RA, 10-10 M</td>
<td>-15</td>
<td>-8</td>
<td>+40</td>
<td>+67</td>
<td>+168</td>
<td>-18</td>
<td>+4</td>
</tr>
<tr>
<td>13-cis RA, 10-8 M</td>
<td>+17</td>
<td>+17</td>
<td>+2</td>
<td>n.d.</td>
<td>-18</td>
<td>0</td>
<td>+21</td>
</tr>
<tr>
<td>13-cis RA, 10-9 M</td>
<td>+6</td>
<td>-2</td>
<td>+5</td>
<td>+20</td>
<td>+4</td>
<td>-29</td>
<td>-11</td>
</tr>
<tr>
<td>13-cis RA, 10-10 M</td>
<td>0</td>
<td>-11</td>
<td>+15</td>
<td>+32</td>
<td>+83</td>
<td>-17</td>
<td>-18</td>
</tr>
</tbody>
</table>

*Quantiﬁcation of changes in keratin expression in human epidermal keratinocytes cultured in the presence of different concentrations of all-trans RA and 13-cis RA as compared to DMSO (control) cultures.

n.d., not detectable.
Our results thus merit additional in vitro and in vivo experiments to obtain further insights into the action of non-aromatic and polyaromatic retinoids.

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