Control of Epidermal Differentiation by a Retinoid Analogue Unable to Bind to Cytosolic Retinoic Acid-Binding Proteins (CRABP)

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The role played by cytosolic retinoic acid–binding proteins (CRABP) in the control of differentiation and morphogenesis by retinoids remains unclear, which contrasts with the presence of these binding proteins in tissues known to be targets for retinoic acid effects. Human epidermis represents a good system to address this question because 1) the effect of retinoids on keratinocyte differentiation is well documented; 2) epidermis contains CRABP, and the amount of these proteins is modulated both by keratinization and retinoids; 3) the architecture of epidermis obtained in vitro by growing adult human keratinocytes on a dermal substrate can be modulated by retinoids added to the culture medium in a dose-dependent manner; and 4) most markers of epidermal differentiation are also modulated by retinoids in a dose-dependent manner.

In this study, we compared, in dose-response experiments, the biologic activities of retinoic acid and CD271, a substance unable to bind to CRABP, but able to bind to nuclear retinoic acid receptors (RAR). Our results show that retinoic acid and CD271 exert similar controls on epidermal morphogenesis and keratinocyte differentiation, as shown by the inhibition of the synthesis of suprabasal keratins, filaggrin, and transglutaminase. Therefore, we exclude a qualitative role for CRABP in the control exerted by retinoids on the differentiation and morphogenesis of cultured human keratinocytes. Instead of being involved in the pathway via which retinoids control epidermal gene expression, CRABP might regulate the amount of intracellular-active retinoic acid and thus control quantitatively the intensity of biologic effects. *J Invest Dermatol 98:128–134, 1992*

Retinoic acid (RA) is a morphogenetic hormone produced in vertebrates after metabolization of dietary vitamin A and carotenoids [1,2]. This mediator plays a major role in the differentiation and morphogenesis of many tissues [3–6]. For instance, RA has been shown to exert a stringent control on the differentiation of epithelia, both in the embryo and in the adult [7–9]. Moreover, it would appear that, in vivo, a critical concentration of retinoids is required for appropriate differentiation of epidermis as suggested by the existence of cutaneous symptoms in hyper- and hypovitaminosis A syndromes [7,8,10]. In vitro, RA inhibits the synthesis of most epidermal differentiation markers in a dose-dependent manner [11–16]. Moreover, in a tissue culture system that reconstructs epidermis, a normally differentiated (i.e., orthokeratotic) epithelium is obtained at low RA concentrations, whereas higher concentrations lead progressively to the disappearance of stratum granulosum and stratum corneum (parakeratosis) [14].

The biologic effects of RA on gene expression are mediated by binding to nuclear receptors [17] belonging to the steroid-thyroid hormone receptor family [18,19]. After binding the ligand, the receptors are able to modulate the transcription of specific genes by interacting with responsive elements located in the promoter regions of these genes [20,21]. Up to now, three different RA receptors (RARα, β, γ) showing high sequence similarities have been identified in the human and in the mouse [22]. In the skin of both species, although RARα and RARβ can be detected, RARγ receptors are predominant [22,23]. Moreover, the distribution of RARγ transcripts in mouse embryos as detected by in situ hybridization seems to be uniform in dermis and epidermis [24,25].

Cytosolic RA-binding proteins (CRABP) have been known for a much longer time than RAR, although their role is still unclear [26,27]. Two distinct proteins, CRABP I and CRABP II, showing a high sequence homology, have been described so far [28–31]. In a recent report [17], we have shown that in cultured F9 embryonal carcinoma cells there is no correlation between the biologic activity of a series of natural and synthetic retinoids and their binding to CRABP, although a strong correlation between biologic activity and binding to nuclear receptors was shown. Moreover, retinoids with no affinity for CRABP were synthesized and shown to be biologically active and able to bind to RA nuclear receptors [17,32]. Along the same line, it must be noted that certain cell lines devoid of CRABP are able to respond to RA [33]. This seems to exclude a role for CRABP in mediating RA effects at the level of individual cells, but does not eliminate the possibility that CRABP are involved in pattern formation by creating or intensifying RA gradients within developing tissues [24,34–36].

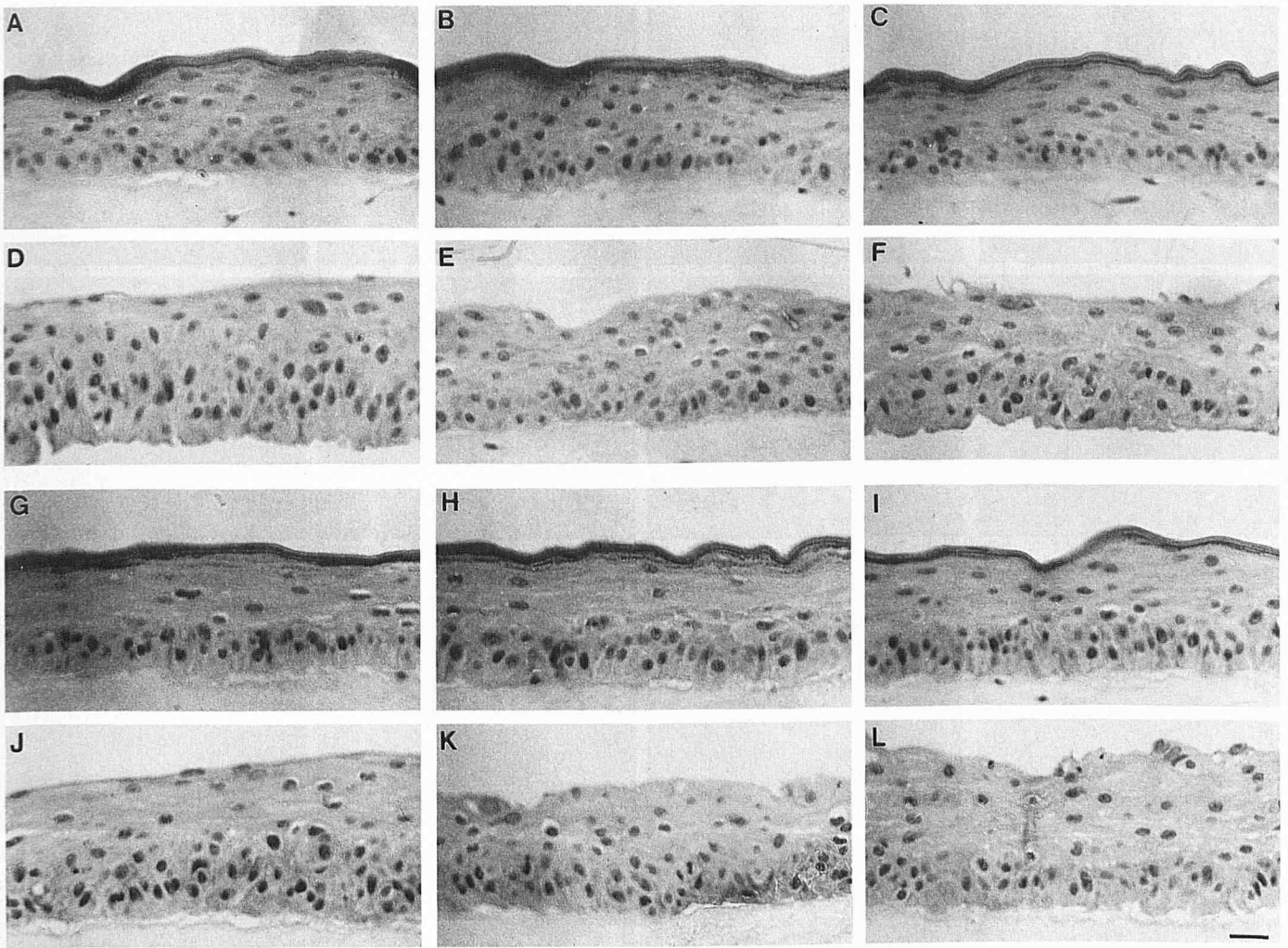CRABP are present in human epidermis [37] and they are de-

Figure 1. Morphology of the epithelium formed by human keratinocytes grown for 2 weeks on a dermal equivalent that was emerged the second week in the presence of added RA *(A,B,C,D,E,F)* or CD271 *(G,H,I,J,K,L)*. Concentrations of either RA or CD271 were as follows: 0 *(A,G)*; 10^{-10} M *(B,H)*; 10^{-9} M *(C,I)*; 10^{-8} M *(D,J)*; 10^{-7} M *(E,K)*; 10^{-6} M *(F,L)*. Granular and horny layers were focused. *(Bar, 30 μm.)*

tected at low levels in undifferentiated cultured keratinocytes, whereas high levels are found in differentiating keratinocytes [38]. Moreover, CRABP levels are increased by treatment with retinoids [28,39]. Thus, it is tempting to hypothesize that CRABP participate in the modulation of the epidermal phenotype exerted by retinoids. However, in the present work, using a culture system enabling the reconstruction of epidermis, we have not detected differences between the biologic activities of RA and CD271, a retinoid unable to bind to CRABP.

MATERIALS AND METHODS

Retinoids All-trans RA came from Interchim (France); CD271 was synthesized according to the procedure described in Shroot et al, 1986 [40]. Contrary to RA, this substance was shown not to bind to the CRABP contained in rat testis protein extracts [41]. Radio-labeled RA (55 Ci/mmol) was purchased from NEN (Dupont de Nemours, Paris, France); CD271 was tritiated (23 Ci/mmol) as described elsewhere [17].

Other Chemicals Dimethyl sulfoxide (DMSO) was purchased from Merck (Germany); epidermal growth factor (EGF) from Collaborative Research (USA); and the other tissue culture supplements from Sigma (USA).

Tissue Culture

Reconstructed Epidermis: Our method for seeding and culturing keratinocytes on fibroblast-collagen lattices was previously described in detail [42]. Briefly, adult interfollicular epidermal cells isolated from human breast skin and amplified in primary culture, are seeded on the lattices. The cultures are first kept submerged for 1 week, then raised at the liquid-air interface during another week. This two-step procedure results in the formation of a stratified and keratinized epithelium. The tissue culture medium used for reconstructed epidermis was minimum essential medium (MEM) supplemented with 10% fetal calf serum, EGF (10 ng/ml), hydrocortisone (0.4 μg/ml), and cholera toxin (10^{-9} M). All-trans RA or CD271, dissolved in DMSO in 1000 times stock solution, were added to the culture medium under yellow light. Control cultures received the same amount of DMSO. Cultures supplemented with retinoids were kept in the dark and the medium was changed three times weekly.

Keratinocyte Cultures in Serum-Free Medium: Keratinocytes grown on plastic were cultured in MCDB153 (KBM, Clonetics Corp, San Diego, CA) as described previously [43].

Histology Histology was performed by staining vertical paraffin sections with hemalum-phloxine-saffron.
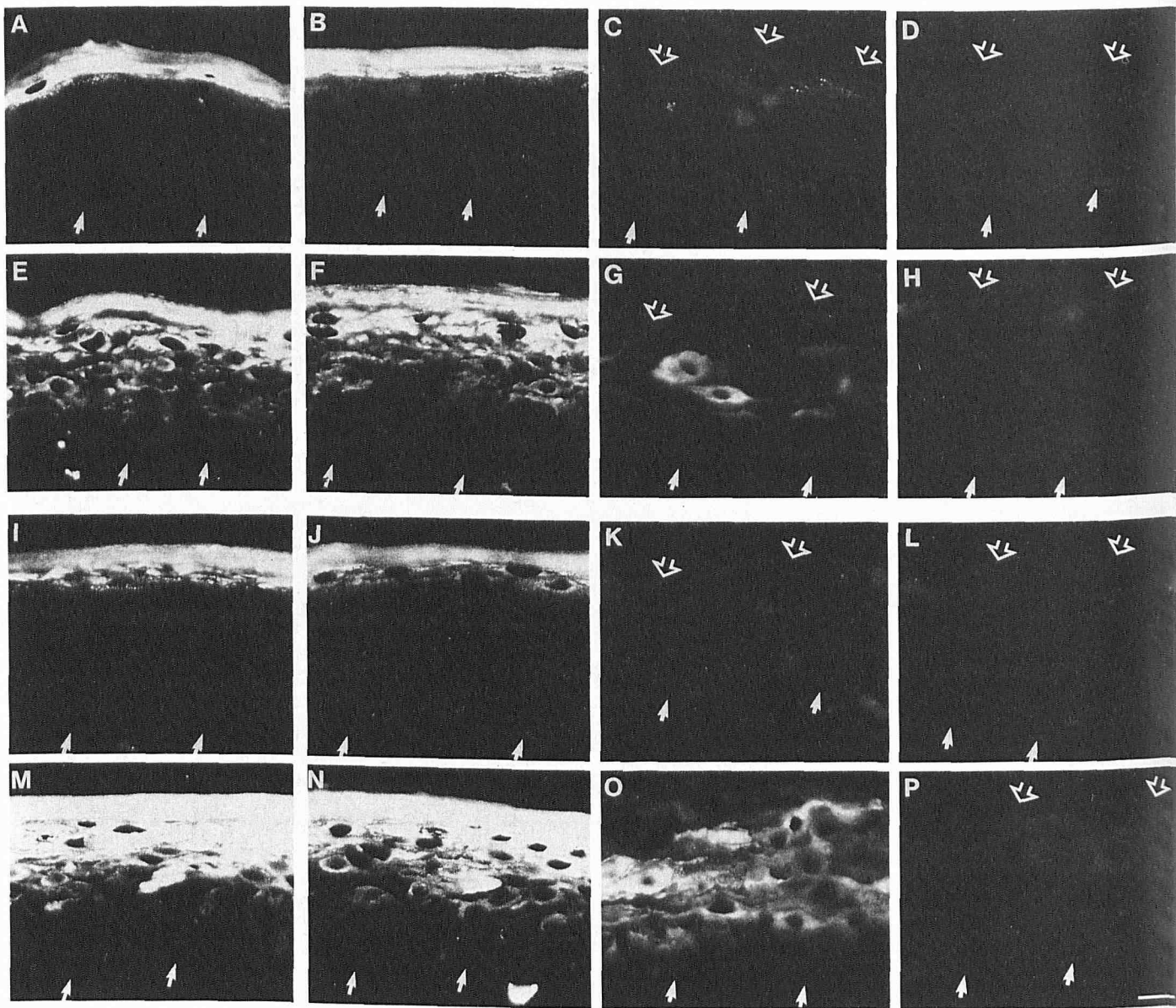
Figure 2. Immunolabeling of filaggrin *(A,B,C,D; I,J,K,L)* and K1/K10 keratins *(E,F,G,H,M,N,O,P)* in the epithelium formed by human keratinocytes grown for 2 weeks on dermal equivalent that was emerged the second week in the presence of added RA *(A,B,C,D,E,F,G,H)* or CD271 *(I,J,K,L,M,N,O,P)*. Concentrations of either RA or CD271 were as follows: 0 *(A,E,I,M)*; 10^{-9} M *(B,F,J,N)*; 10^{-8} M *(C,G,K,O)*; 10^{-7} M *(D,H,L,P)*. *Open arrows* point at the edge of the upper layers and *solid arrows* indicate the junction between the cultured epithelium and the dermal equivalent. *(Bar, 30 μm.)*

Antisera and Immunofluorescence Staining Monoclonal antibody (MoAb) KG8.60 [44] directed against the differentiation-specific K10 keratin was from Bioyeda, Israel. MoAb against human filaggrin [45] was from BTI (Stoughton, MA). The specificity of these antibodies was checked by immunoblotting performed with protein extracts of both human epidermis and cultured human keratinocytes. These experiments revealed that in addition to K10 keratin, KG8.60 MoAb recognizes K1 keratin, the basic suprabasal keratin partner of K10 keratin (D. Asselineau, unpublished observations). Antifilaggrin MoAb reacted against profilaggrin and filaggrin [45].

Indirect immunofluorescence studies on 5-μm frozen tissue sec-

tions were performed as previously published [46] except that bovine serum albumin was replaced by low-fat dried milk (Régilait, France).

ELISA for Epidermal Transglutaminase The enzyme-linked immunosorbent assay (ELISA) to determine epidermal transglutaminase was performed on keratinocytes cultured in defined medium as described [43].

Binding to the CRABP of Human Skin Cells Cultured cells (human fibroblasts and keratinocytes) and reconstructed tissues (epidermis) were homogenized in tris 50 mM pH 7.8 by the nitrogen decompression method (Cell Disruption Bomb, Parr, USA). After
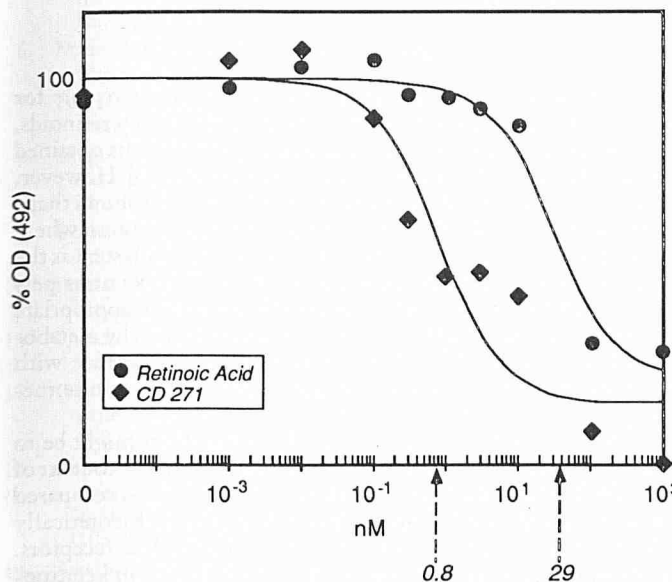
MEMBRANE - BOUND TRANSGLUTAMINASE

Figure 3. Dose-response curves showing the inhibition by RA and CD271 of the production of membrane-bound transglutaminase (Tgm) in cultured human keratinocytes using an ELISA assay (see *Materials and Methods*). Note that the inhibitory concentrations (IC50) leading to a 50% inhibition of Tgm are 29 nM and 0.8 nM for RA and CD271, respectively (for the calculations of IC50, the residual activity at 10^{-6} M was subtracted).

disruption, cells and tissue extracts were centrifuged at $100,000 \times g$ for 20 min. The protein contents of the supernatant were determined by the Biorad assay. Using the supernatant (50 μg protein), binding to cytosolic retinoic acid – binding proteins was performed and analyzed, using gel filtration as described [41]. Because this technique does not discriminate between CRABP I and CRABP II, we used the term CRABP to define a pool of cytosolic retinoic acid – binding proteins of approximately 15 kD molecular weight able to bind RA and some synthetic retinoids with high affinity [41].

Binding to Human α, β, and γ RAR Binding assays were performed on nuclear extracts of Cos7 cells transfected with vectors expressing RARα [47], RARβ [48], or RARγ (purchased from La Jolla Cancer Research Foundation) as described [49].

Production and Extraction of CD271 Radio-Labeled Metabolites in Culture

[³H] CD271 Uptake in Culture: Radio-labeled CD271 was added to the culture medium to reach a concentration of 10^{-7} M of total labeled and cold CD271. It was added at the time the cultures were raised at the liquid-air interface and re-added at each medium change. Thus, the cultures were incubated with the radio-labeled retinoid during the whole time necessary to reconstruct epidermis in vitro. All tissue culture media fractions were pooled and stored at $-70°$C together with the reconstructed tissues obtained at the end of the culture.

Extraction of the Pool CD271-CD271 Metabolites: The reconstructed epidermis and the dermal substrates (fibroblast-contracted collagen lattices) were homogenized and sonicated in the pool of labeled tissue culture medium. This preparation was submitted to a triple extraction cycle as follows: 5 volumes of ethyl acetate were added to the homogenate; the mixture was submitted to rotary shaking at 37°C for 1 h 30 min and centrifuged at 3,000 rpm for 10 min (Heraeus centrifuge). At the end of the extraction steps, all supernatants were pooled and evaporated. The pellets were resuspended in DMSO. This procedure yielded 95% of the initial labeling added to the tissue culture medium. High-performance liquid

Table I. Binding of CD271 and RA to Human Nuclear Retinoic Acid Receptors[a]

	RARα[b]	RARβ[b]	RARγ[b]
RA	13	3	3
CD271	1100	34	180

[a] Kd of RA and CD271 for RARα, β, and γ. For details, see *Materials and Methods* and [45]. The values shown correspond to representative experiments.
[b] Kd (nM).

chromatography (HPLC) control analysis showed that approximately 17% of the radio-labeled CD271 was converted into metabolites. The pool of radio-labeled CD271 and CD271 metabolites was then used for examining its ability to bind to CRABP as described above.

RESULTS

CD271 Binds to Human Retinoic Acid Receptors RARα, β, and γ, but Not to CRABP CD271, a retinoic acid analogue able to induce differentiation of F9 embryonal carcinoma cells, was previously found to be able to bind to F9 nuclear retinoic acid receptors, but not to rat testis CRABP [17,41]. Before comparing the biologic activities of CD271 and RA on epidermal differentiation, we checked that CD271 was able to bind to human nuclear retinoic acid receptors RARα, β, and γ (Table I), but not to CRABP isolated from human keratinocytes or fibroblasts grown in tissue culture. The data summarized in Table I show that CD271 binds to α, β, and RARγ, although with an affinity 10–80 times lower than that of RA. In contrast, it was not possible to detect any specific binding of CD271 on cytosolic extracts of keratinocytes or fibroblasts, although these cells contained CRABP as shown by the specific binding of RA on a 15 kD cytosolic fraction (Table II). The kD of RA for these human CRABP were approximately 2 nM, a value similar to that reported for rat CRABP [41].

RA and CD271 Exert Similar Morphogenetic Effects on Human Epidermis Cultured In Vitro Normal human keratinocytes were grown on fibroblast-collagen substrates at the air-liquid interface, conditions known to lead to the formation of a multilayered epidermis whose phenotype is dependent on the retinoid concentration present in the medium [14]. In this work, the effects of serial concentrations of RA and CD271 on the architecture of the cultured epidermis were compared. Results reported in Fig 1 show that the response of the cultured epidermis to increasing retinoid concentrations was qualitatively and quantitatively similar for RA

Table II. Specific Binding of RA to the CRABP[a]

Specific Binding to CRABP Prepared From	RA	CD271	CD271 Metabolic Pool
GM10 human fibroblasts cultured on plastic	5 pmol/mg protein	nd	
Normal human keratinocytes cultured on plastic	1.2 pmol/mg protein	nd	nd
GM10 human fibroblasts incorporated in the collagen lattice (dermal equivalent)	2.7 pmol/mg protein	nd	
Normal human keratinocytes in the reconstructed epidermis	10.7 pmol/mg protein	nd	nd

[a] CRABP contained in cytosolic extracts prepared from GM10 human fibroblasts and normal human keratinocytes, either cultured on plastic or incorporated in the dermal equivalent and the reconstructed epidermis, respectively. No specific binding (nd, not detectable) was found in similar experiments performed with both CD271 and the CD271 metabolic pool. For details, see *Materials and Methods* and [37]. The values shown correspond to representative experiments.

and CD271. In the absence of any added retinoid, the epithelium grown in non-delipidized serum was apparently normal, as previously described [14,46]. Upon additions of up to 10^{-9} M RA or CD271 to the medium, the epidermis remained orthokeratotic (see Fig 1A,B,C, and G,H,I, respectively), but no stratum corneum (parakeratosis) could be observed at RA and CD271 concentrations of 10^{-8} M and above (see Fig 1D,E,F and J,K,L, respectively).

Similar Effects of RA and CD271 on K1/K10 Keratin and Filaggrin Distribution Within Cultured Epidermis

In vivo, K1–K10 keratins appear during the first stages of epidermal differentiation (spinous layers) [50], whereas filaggrin is produced during the last steps of the keratinization process [51]. We have previously shown that in the reconstructed epidermis, at physiologic retinoid concentrations, K1/K10 keratins also appear early during epidermal differentiation in the suprabasal (spinous) layers, whereas filaggrin is seen later in granular layers [14]. These markers are down-regulated by RA [13,14,51]. A representative example of the effects of RA and CD271 on the patterns of K1/K10 keratins and filaggrin distribution is shown in Fig 2. Upon additions of up to 10^{-9} M RA or CD271, both K1/K10 keratins and filaggrin retained their normal location in the epithelium formed (Fig 2A,B,E,F and I,J,M,N, respectively). Filaggrin was restricted to a relatively thin granular layer, whereas K1/K10 keratins were distributed in most of the suprabasal layers. At 10^{-8} M added RA or CD271, filaggrin was not detectable (Fig 2C and K, respectively), whereas K1/K10 keratins were present, but in reduced amounts, in suprabasal cells (Fig 2G and O). At 10^{-7} M added RA or CD271, neither filaggrin nor K1/K10 keratins could be detected (Fig 2D,H and L,P, respectively).

CD271 Metabolites Do Not Bind to CRABP

A trivial explanation of our results would have been that CD271 is converted by keratinocytes into metabolites able to bind to CRABP. It was crucial to eliminate this possibility, because CD271 is actually metabolized by a variety of cell types, including keratinocytes (C. Filaquier, personal communication). Labeled CD271 was incubated in culture during the time necessary for reconstruction of epidermis (see *Materials and Methods*) at a concentration of 10^{-7} M, known to provoke maximal biologic effects (see above). The pool of CD271 and CD271 metabolites was extracted as described in *Materials and Methods* at the end of the complete time course of the culture, and assayed for binding to CRABP isolated from keratinocytes grown either on plastic or on lattices (reconstructed epidermis). No specific binding could be found with CD271 and the CD271 metabolic pool (Table II). These experiments also showed that CD271 was actually metabolized (17% of the added CD271 was converted into three different metabolites, C. Filaquier, personal communication) and that the histology of the reconstructed epidermis was similar to that obtained with 10^{-7} M unlabeled CD271.

CD271 and RA Are Both Able to Control the Differentiation of Keratinocytes Grown in Serum-Free Medium

Experiments in which epidermis is reconstructed in vitro are important for the study of retinoid effects on the morphogenesis and differentiation of the whole tissue. However, it might be delicate to interpret the results obtained in this system, because cultures are performed in the presence of 10% fetal calf serum (FCS), which already contains natural retinoids, mainly retinol. In this context, the biologic effects of CD271 could be interpreted as the result of an interference with the metabolism of endogenous retinoids. For instance, it could be argued that CD271 is able to induce the transformation of retinol into RA or other retinoids able to bind to CRABP. The culture of human keratinocytes in a serum-free medium containing no retinol (MCDB153) allowed us to eliminate this possibility. Figure 3 shows the dose-dependent inhibitions by RA and CD271, added to keratinocytes cultured in MCDB153, of the membrane-bound transglutaminase, an enzyme induced during epidermal differentiation. The IC50 of CD271 was 0.8 nM, whereas the IC50 of RA was 29 nM. Thus, in this system, CD271 was much more potent than

RA, a finding that contrasts with its weaker affinity for RAR (see Table I and *Discussion*).

DISCUSSION

The results reported above seem to exclude a qualitative role for CRABP in the control of keratinocyte differentiation by retinoids, at least in vitro. They are in good agreement with results obtained with embryonal carcinoma cells [17] and HL60 cells [33]. However, in addition, the use of cultured reconstructed epidermis shows that a requirement for CRABP cannot even be shown in a system where morphogenesis is coupled to differentiation. Even details such as the higher sensitivity of filaggrin compared to K1/K10 keratins persisted when CD271 was used instead of RA. Moreover, appropriate controls showed that CD271 effects were not mediated by metabolites able to bind to CRABP, or because of an interference with retinoids contained in the serum-supplemented tissue culture medium.

The role of CRABP remains open. Their functions might be to stabilize or protect RA from degradation enzymes. In the context of this hypothesis, the biologic activity of CRABP ligands compared to that of retinoids unable to bind to CRABP, should theoretically be higher than expected from their affinity for nuclear receptors. However, this is neither the case in F9 cells [17], nor in keratinocytes (the present study). The affinity of CD271 for RAR was 10–80 times lower than that of RA, but its activity was either equal to (K1/K10 keratins, filaggrin) or even higher (transglutaminase) than that of RA. The discrepancy between the higher affinity of RA for the RAR and its weaker biologic activity suggests that CRABP may act by decreasing the level of intracellular-active RA. Although other explanations for this discrepancy can be proposed, e.g., that CD271 might be more stable than RA, or that binding of CD271 to RXR [52] might be better than that of RA, the hypothesis that CRABP act by reducing the intensity of RA effects fits well with two lines of results: 1) in embryonal carcinoma cells engineered to contain varying levels of CRABP I, overexpression of the binding protein was shown to result in a reduction of RA effects on gene expression [53]; and 2) the induction of CRABP II by retinoids [28,39] might be understood as a feedback mechanism to attenuate the biologic effect of increased retinoid concentrations. This might be particularly required in epidermis and other stratified epithelia, in which RA plays essentially a negative role on differentiation. In this respect, it is particularly interesting to note that CRABP levels have been reported to be increased when keratinocytes are not completely differentiated as in oral mucosa, psoriatic plaques, or in tissue culture [34,37,54]. At any rate, our results stress the importance of reevaluating with compounds such as CD271 the assumption that CRABP are involved in the formation of RA gradients in the development of the limb bud [55,56] and possibly other complex biologic structures.

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ANNOUNCEMENT

The 41st Annual Symposium on the Biology of the Skin will be held at Snowmass Village, Colorado, July 25–29, 1992. The symposium, this year on the "Fundamentals of Hair Biology," will be organized by Kirk Wuepper, Andrew Messenger, and David Norris. A program of formal presentations and a limited number of poster presentations are planned. Abstracts for selection of poster presentations should be submitted by May 1, 1992. For details please contact David A. Norris, M.D., Department of Dermatology, B-144, University of Colorado School of Medicine, 4200 East Ninth Avenue, Denver, Colorado 80262. Telephone: (303) 372-1140. Fax: (303) 372-1159.

ANNOUNCEMENT

The 3rd Asian Dermatological Congress will be held January 15–17, 1993 at the Hong Kong Convention and Exhibition Centre. A joint venture of the Hong Kong Society of Dermatology & Venereology and the Asian Dermatological Association, this Congress is being organized by the Institute for International Research. Congress Manager: IIR Ltd., Room 1804-5, Seaview Commercial Building, 21-24 Connaught Road West, Hong Kong. Tel: 549-5618. Fax: 548-7235.