A Limited Role for Retinoic Acid and Retinoic Acid Receptors RARα and RARβ in Regulating Keratin 19 Expression and Keratinization in Oral and Epidermal Keratinocytes

Margarete Schön and James G. Rheinwald

Division of Dermatology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, U.S.A.

Different types of stratified squamous epithelia—for example, the "orthokeratinized" epidermis, the "parakeratinized" gingiva, and the "nonkeratinized" oral lining mucosal epithelia—are formed by intrinsically distinct keratinocyte subtypes. These subtypes exhibit characteristic patterns of keratin protein expression *in vivo* and in culture. Keratin 19 is an informative subtype-specific marker because the basal cells of only nonkeratinizing epithelia express K19 *in vivo* and in culture. Epidermal keratinocytes normally do not express K19, but can be induced to do so in culture by retinoic acid (RA). Keratinocyte subtypes express the retinoic acid receptor (RAR)β at levels roughly correlated with their level of K19 expression in culture and their potential for forming a nonkeratinized epithelium *in vivo*. We tested the hypothesis that the level of RARβ expressed by a keratinocyte determines its K19 expression and its form of suprabasal differentiation. Normal human epidermal and gingival keratinocytes stably overexpressing either RARβ or RARα were generated by defective retroviral transduction. Overexpression of either receptor enhanced the RA inducibility of K19 in conventional culture, in that the proportion of the transductants becoming $K19^+$ in response to RA was markedly increased compared with controls. The pattern of differentiation of the epithelium formed in organotypic culture, assessed by basal K19 and suprabasal K1, K4, and filaggrin expression, however, was unaltered by RAR overexpression. Thus, the susceptibility of keratinocytes to regulation of K19 expression by retinoids is conditional, and levels of neither RARβ nor RARα are limiting to the intrinsic mechanism that specifies alternate differentiation pathways for stratified squamous epithelia. ***Key words: cell culture/epithelial/organotypic/transfection. J Invest Dermatol 107:428–438, 1996***

The surfaces of the body and of the contiguous orifices, including the oral cavity, are covered by stratified squamous epithelia. In these renewal tissues, cells from the basal, proliferative layer commit to terminal differentiation, migrate upward, and express specialized proteins responsible for their morphologic and functional differentiation. The histology and the structural macromolecules of the suprabasal cells differ among these epithelia with respect to their location and function, such as whether they form a barrier against drying, whether they maintain a layer of mucopolysaccharides on their surface, and the amount of abrasion they experience. Classically, two main types of stratified squamous epithelial differentiation have been recognized: "keratinizing" (the extreme example is the epidermis) and "nonkeratinizing" (the extreme example is the soft palatal epithelium). These alternate forms of histogenesis are associated with the suprabasal expression of different keratin proteins and the presence or absence of filaggrin, a keratin filament packing protein (reviewed by Galvin *et al*, 1989; Dale *et al*, 1990). The basal keratinocytes of all stratified squamous epithelia express the keratin pair K5/K14 (Nelson and Sun, 1983). Upon entering the suprabasal compartment, epidermal keratinocytes express K1/K10 and filaggrin, whereas nonkeratinizing oral keratinocytes express K4/K13 and do not express filaggrin. The "parakeratinizing" epithelium of the hard palate and gingiva expresses both K1/K10 and K4/K13 suprabasally (Reibel *et al*, 1989). Basal keratinocytes of nonkeratinizing oral epithelia express K19 in addition to K5 and K14; basal cell K19 is always predictive of suprabasal K4/K13 *in vivo* (Bartek *et al*, 1986; Morgan *et al*, 1987; Lindberg and Rheinwald, 1989, 1990).

Pure populations of normal human keratinocytes cultured from various stratified squamous epithelia have been studied for their expression of differentiation-related proteins, both in culture and after transplantation back to an experimental *in vivo* environment (Doran *et al*, 1980; Wu *et al*, 1982; Lindberg and Rheinwald, 1990). These experiments revealed that stratified squamous epithelia are formed by intrinsically distinct keratinocyte subtypes, each of which has been imprinted during embryogenesis to express only one of several possible programs of differentiation under normal conditions. Although the conditions of conventional culture do not favor

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Reprint requests to: Dr. James G. Rheinwald, Division of Dermatology, Thorn 805, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.

Abbreviations: RA, all-trans retinoic acid; [RA], RA concentration.

expression of most suprabasal differentiation proteins (Fuchs and Green, 1981), keratinocytes of different subtypes express characteristic levels of K19 in culture: Epidermal and gingival keratinocytes express little or no K19, whereas floor-of-mouth and buccal mucosal keratinocytes express moderate levels and soft palatal keratinocytes express high levels of K19 (Lindberg and Rheinwald, 1990; Oda *et al*, 1990).

The mechanism responsible for specifying distinctive patterns of K19 expression and programs of suprabasal differentiation by keratinocyte subtypes remains obscure. Classic dietary deficiency studies and subsequent studies of epidermal keratinocytes in culture (see Fuchs and Green, 1981; Eckert and Green, 1984; Gilfix and Eckert, 1985; Kopan *et al*, 1987; Fuchs, 1990) showed that stratified squamous epithelial differentiation and K19 expression are subject to regulation by the vitamin A metabolite all-trans retinoic acid (RA). The biologic effects of RA are now known to be mediated by six different nuclear hormone receptors: RARα, RARβ, RARγ, and RXRα, RXRβ, and RXRγ. A direct mechanism of gene regulation by RA and these receptors has been characterized in detail. RARs form heterodimers with RXRs and bind to retinoic acid response elements—short, direct repeats of DNA sequences similar to 5′-AGTTCA-3′—that are found upstream of the promoters and transcription start sites of many RA-responsive genes (reviewed by Chambon, 1994; Pfahl, 1994). Co-repressors and co-activators that modulate the activity of RA receptor/retinoic acid response element complexes have been discovered very recently (Kurokawa *et al*, 1995). Interestingly, many keratin genes, including K19, do not contain recognizable retinoic acid response elements (Lussier *et al*, 1989; Bader and Franke, 1990; Winter *et al*, 1994), although an unconventional RA response sequence has been identified recently in the K6 gene (Navarro *et al*, 1995). Transient transfection experiments have not identified any sequence upstream or downstream of the K19 coding region that can mediate RA inducibility (Hu and Gudas, 1994). Thus, the mechanism by which RA can regulate the expression of K19 remains to be determined. It may involve inhibition of AP1 transcriptional regulatory proteins by RA-receptor complexes, an indirect mechanism that has been identified as part of the regulatory system of other genes (Nicholson *et al*, 1990).

An earlier study found that RARγ is expressed abundantly by all keratinocyte subtypes and that all subtypes express lower, but also equal, levels of RARα. In contrast, a great range of RARβ expression was found among keratinocyte subtypes, correlating roughly with their level of K19 expression in conventional culture and with expression of a nonkeratinizing type of suprabasal differentiation *in vivo* (Crowe *et al*, 1991; Hu *et al*, 1991; Xu *et al*, 1994). This suggested to us a two-part hypothesis: (i) that RARβ is responsible for regulating K19 expression in keratinocytes, and (ii) that RARβ is the limiting factor in the mechanism that specifies a nonkeratinizing, as opposed to a keratinizing, form of suprabasal differentiation. We report here the results of testing these hypotheses by examining the properties of epidermal and gingival keratinocytes genetically modified by stable retroviral transduction to overexpress RARβ or RARα.

MATERIALS AND METHODS

Cells and Culture Methods The derivation and characteristics of the normal human keratinocyte cell strains used were described previously (Lindberg and Rheinwald, 1990; Crowe *et al*, 1991). Strains, keratinocyte subtypes, and tissues of origin, respectively, were as follows: strain N, epidermal orthokeratinizing, foreskin epidermis; B1-Ep, epidermal orthokeratinizing, interfollicular epidermis; OKG4, oral parakeratinizing, gingival epithelium; OKF4, typical oral nonkeratinizing, floor-of-mouth epithelium; and OKP7, special oral nonkeratinizing, soft palatal epithelium.

Cells were thawed from cryopreserved second- or third-passage stocks and cultured in GIBCO keratinocyte serum-free medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 50 μg bovine pituitary extract per ml, 0.1 ng epidermal growth factor per ml, and $CaCl_2$ to bring the total Ca^{2+} concentration to 0.4 mM.

For analysis in conventional (i.e., plastic substratum, submerged) culture, cells were plated at densities of 5×10^3 to 10^4 per 9-cm^2 well in six-well plates or at 10^5 cells per T25 flask in keratinocyte serum-free medium and grown for 3–4 d. At this time, cultures were refed, and some received RA (Sigma Chemical Co., St. Louis, MO) for the final 2 d of growth. Concentrated stock solutions of RA in dimethylsulfoxide (DMSO), 1000X, were diluted into the culture medium. Control cultures received 0.1% DMSO.

Organotypic cultures were prepared as described previously (Parenteau *et al*, 1991; Parenteau, 1994). Briefly, a solution of bovine type I collagen (Organogenesis, Inc., Canton, MA) was allowed to gel with 2.3×10^4 human foreskin dermal fibroblasts (strain B038) per ml of gel in six-well tissue culture tray inserts having a 3-μm–pore polycarbonate membrane base. A 1-ml layer of acellular collagen gel was first cast on the membrane, and then a 3-ml collagen layer containing the fibroblasts was cast on top of it (the collagen concentration of these gels was 0.7 mg per ml). The embedded fibroblasts contracted the collagen gels during a 4-d incubation at 37°C. Keratinocytes were then seeded onto the gels at a plating density of 2×10^5 cells per ~1 cm^2 surface area of each contracted gel and were cultured for 4 d submerged in medium consisting of Dulbecco's modified Eagle's medium/F12 (3:1 v:v) supplemented with 0.3% bovine serum, 5 μg insulin per ml, 0.4 μg hydrocortisone per ml, 20 pM triiodothyronine, 5 μg transferrin per ml, 10^{-4} M ethanolamine, 10^{-4} M phosphoethanolamine, 5.3×10^{-8} M selenious acid, and 1.8×10^{-4} M adenine. The cultures were then raised to the air-liquid interface for 10 d, with medium changes on days 0, 4, and 8. RA was added at a final concentration of 3×10^{-10} M or 3×10^{-8} M, beginning when the cultures were raised to the air-liquid interface.

Defective Retrovirus-Mediated Gene Transduction Keratinocytes were stably transduced with the defective retroviral vector LXSN (Miller and Rosman, 1989) into which RARα or RARβ cDNA sequences were inserted downstream from the strong promoter/enhancer of the 5′ long terminal repeat (Robertson *et al*, 1992). Preconfluent cultures of PA317 packaging cells (Miller and Buttimore, 1986), producing amphotropic transducing retroviruses, were fed with keratinocyte serum-free medium overnight. Preconfluent keratinocyte cultures were fed with this 0.45-μm–filtered viral supernatant medium overnight in the presence of 4 μg polybrene per ml. Stable transductants were selected by growth in the presence of 0.2 mg (active drug) G418 per ml (Geneticin; Life Technologies).

Antibodies The following mouse monoclonal antibodies and rabbit polyclonal antisera (antigen name, antibody, source, and reference, respectively) were used for Western blotting, fluorescence-activated cell sorter (FACS) analysis, and avidin-biotin complex peroxidase staining: K5, AE14 (T.T. Sun) (Lynch *et al*, 1986); K1/K10, AE2 (T.T. Sun) (Tseng *et al*, 1982); K13, AE8 (T.T. Sun) (Dhouailly *et al*, 1989); K4, 6B10 (Sigma Chemicals, Inc.) (van Muijen *et al*, 1986); K19, K_s19.1 (ICN Biomedicals, Inc.) (Karsten *et al*, 1985); filaggrin, AKH1 (B.A. Dale) (Dale *et al*, 1987); RARα, no. 115/RPα(F) (P. Chambon) (Gaub *et al*, 1992); and RARβ, no. 112/RPβ(F)2 (P. Chambon) (Rochette-Egly *et al*, 1992).

The RARα and RARβ antisera were raised against unique peptide sequences in the C terminal, F domains of these receptors. As such, each antiserum recognizes all isoforms of the respective receptor type resulting from alternative splicing in the A region (Gaub *et al*, 1992; Rochette-Egly *et al*, 1992).

Western Blotting Nuclear and cytoskeletal extracts from conventional and organotypic cultures were prepared as described previously (Andrews and Faller, 1991; Wu *et al*, 1982). Fifteen micrograms of protein from the 0.4 M NaCl-soluble "nuclear extract" or from the Triton/0.4 M NaCl-insoluble "cytoskeletal fraction" was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. RARα and RARβ were detected with receptor type–specific, anti-peptide rabbit antisera, and K19 with mouse monoclonal antibody K_s19.1. Peroxidase-labeled, goat anti-rabbit or anti-mouse IgG secondary antibodies and a chemiluminescence reaction (ECL; Amersham Corp., Arlington Heights, IL) were used to detect the proteins on blots.

Fluorescence-Activated Cell Sorting Cultured cells were suspended with trypsin/ethylenediamine tetraacetic acid, fixed with 1% paraformaldehyde in phosphate-buffered saline, and permeabilized with 0.3% saponin. After rinsing with phosphate-buffered saline, the cells were incubated with K19- or K14-specific mouse monoclonal antibody or nonimmune mouse IgG and then with a fluorescein isothiocyanate–conjugated, goat anti-mouse IgG secondary antibody. Approximately 10^5 cells of each sample were analyzed by FACS (Becton-Dickinson, Inc., San Jose, CA) using Cell Quest software (BD Immunocytometry Systems, San Jose, CA).

Immunocytochemical and Immunohistochemical Staining Five-micrometer cryosections of organotypic cultures were fixed in cold acetone, air-dried, and stained by the avidin-biotin complex peroxidase technique (Vector Laboratories, Burlingame, CA) using the peroxidase substrate

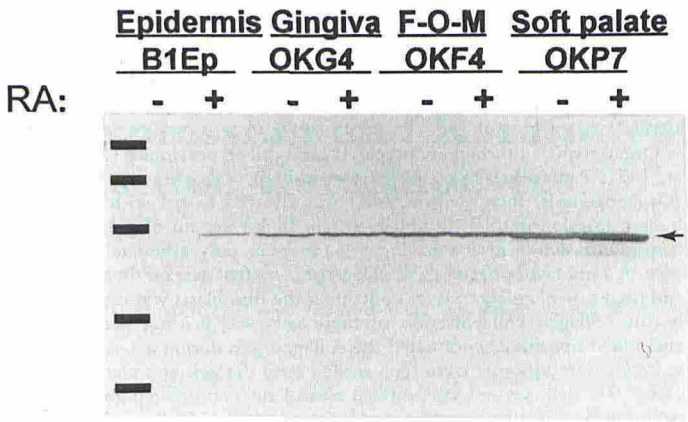

Figure 1. Keratinocytes cultured from epidermis, gingiva, floor of mouth (F-O-M), and soft palate express progressively higher constitutive and RA-inducible levels of K19. Cultures of the indicated normal human epidermal and oral keratinocyte strains received 0.1% DMSO as a control *(−)* or 10^{-7} M RA *(+)* for the final 2 d before extraction. The cytoskeletal fraction was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, and immunoperoxidase stained with a K19-specific antibody. *Marks at left*, positions of molecular weight standards, from top to bottom: 97 kDa, 68 kDa, 43 kDa, 29 kDa, and 18 kDa. *Arrow* points to the position of K19, which migrates in this system as a 40-kDa protein.

3-amino-9-ethyl-carbazole as chromogen and hematoxylin (Gill's formulation; Fisher Scientific, Springfield, NJ) as counterstain. Cultured cells were fixed in their vessels in cold (−20°C) methanol, air-dried, and immunostained using the same methods, without a final counterstain.

RESULTS

Subtype Differences in Constitutive and RA-Induced K19 Expression Are Associated With Different Proportions of K19$^+$ Cells in Phenotypically Heterogeneous Cultures

The relative levels of K19 expressed in culture by human keratinocyte cell strains representing four oral and epidermal subtypes were assessed by Western blotting **(Fig 1)**. Increasing levels of K19 were expressed by the subtypes in the order of decreasing keratinization potential, from orthokeratinizing (interfollicular epidermal strain B1Ep), parakeratinizing (gingival strain OKG4), typical nonkeratinizing (floor-of-mouth strain OKF4), to special nonkeratinizing (soft palatal strain OKP7). All subtypes responded to a 2-d treatment with 10^{-7} M RA by an increase in K19 content. These results are consistent with those of earlier studies comparing K19 protein synthesis rates and mRNA levels of keratinocyte subtypes cultured in the serum-supplemented, feeder layer system (Wu *et al*, 1982; Lindberg and Rheinwald, 1990; Crowe *et al*, 1991).

We then used FACS to quantify K19 expression at the level of single cells, comparing the K19 contents of keratinocyte subtypes under control conditions ($<10^{-10}$ M RA) and after 2 d exposure to a high concentration (10^{-7} M) of RA **(Fig 2)**. Almost all cells in cultures of the epidermal keratinocyte strain N were K19$^-$. A 2-d exposure to high [RA] typically induced less than 10% of the cells to become K19$^+$. Few (<10%) of the cells in gingival keratinocyte cultures were K19$^+$ under control conditions; however, in contrast to epidermal cells, RA treatment typically induced ~30% of gingival cells to express K19. In floor-of-mouth keratinocyte cultures, more than 50% of the cells were K19$^+$ under control conditions and more than 90% became K19$^+$ in response to RA. Soft palatal keratinocytes, identified earlier as a "special nonkeratinizing" subtype distinct from keratinocytes of other nonkeratinizing oral regions (Lindberg and Rheinwald, 1990), were nearly all K19$^+$ in low [RA] and did not acquire a detectably higher K19 content per cell as a result of exposure to RA. The biphasic distributions of K19 content indicate that keratinocytes tend to exist either in a K19$^-$ or a K19$^+$ state. The fluorescence intensities of the

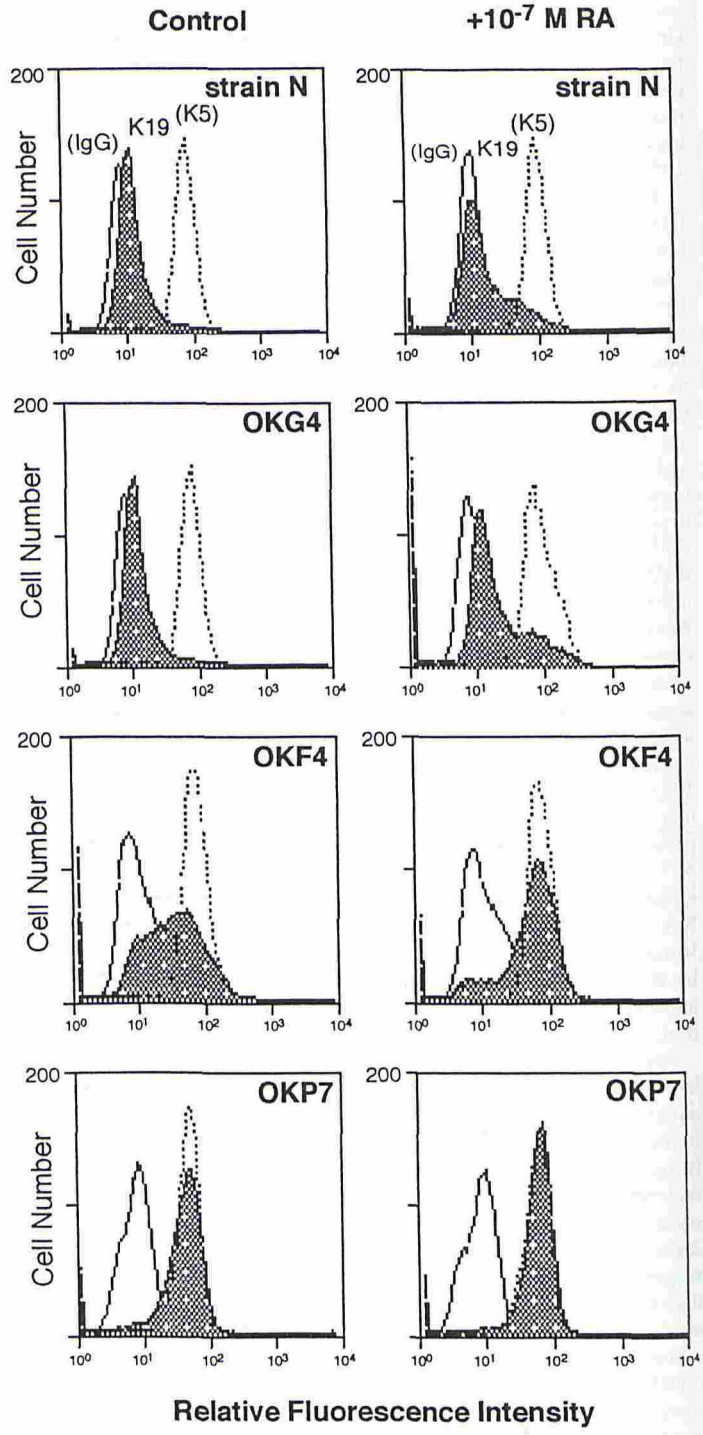

Figure 2. FACS analysis reveals substantial heterogeneity of constitutive and RA-induced K19 expression among cells in cultures of all keratinocyte subtypes. Cells received 0.1% DMSO (control, *left*) or 10^{-7} M RA *(right)* for the final 2 d of culture and then were analyzed for keratin content by FACS. The graphs show frequency distributions of cell fluorescence intensity. *Solid outline*, nonimmune mouse IgG primary antibody; *dotted outline*, K5 antibody; *filled curve*, K19 antibody.

K19$^+$ peaks showed that the maximum K19 content per cell attained by all four subtypes was about the same.

Many small, proliferative cells in nonkeratinizing oral keratinocyte cultures were constitutively K19$^+$, and nearly all the cells expressed K19 in response to a 2-d exposure to RA **(Fig 3*E–H*)**. In contrast, in cultures of epidermal and gingival keratinocytes, only a fraction of the large, flat cells **(Fig 3*A,C*)**, which are postmitotic

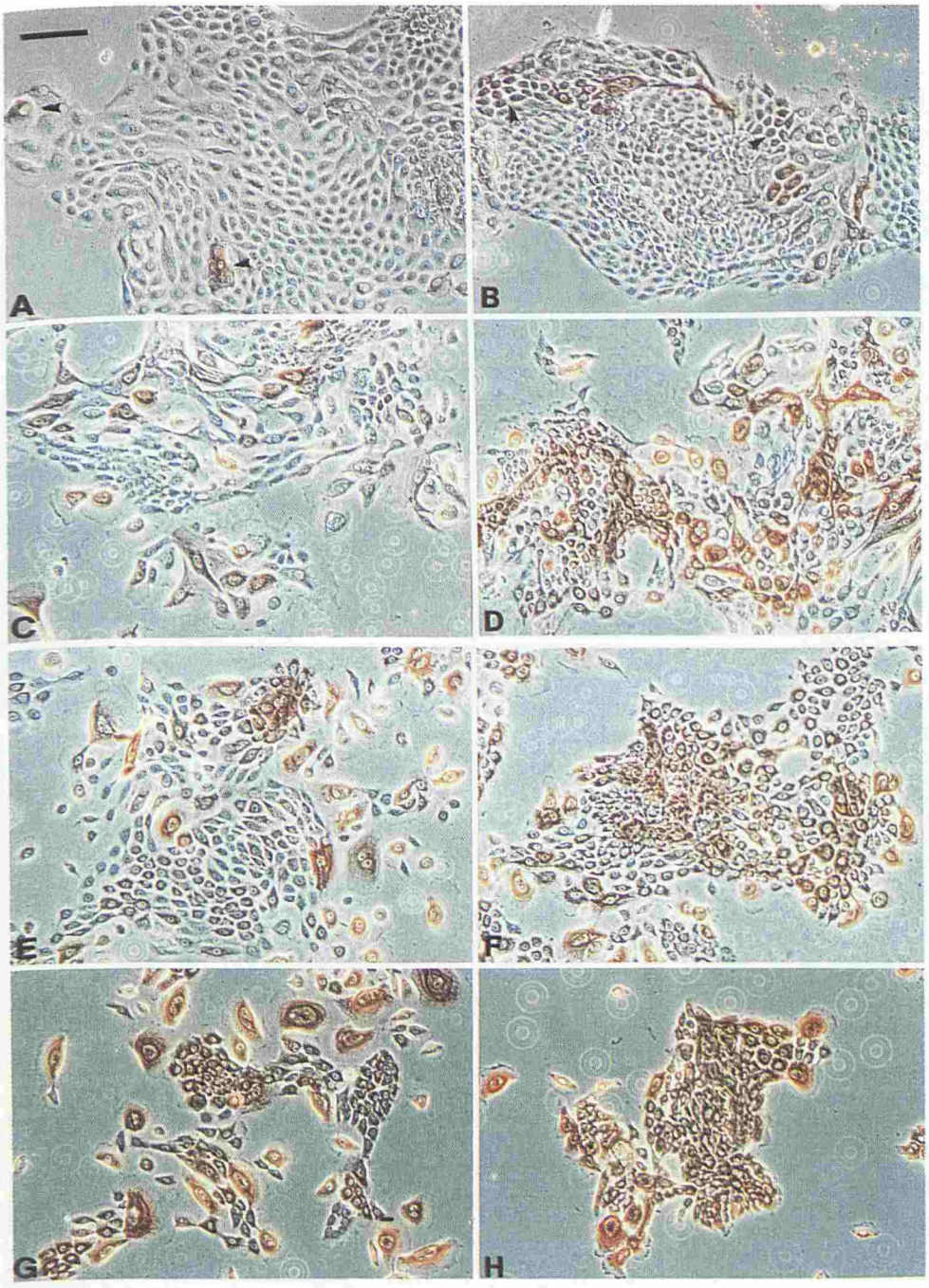

Figure 3. Immunocytochemical staining reveals heterogeneous K19 expression within keratinocyte colonies, with constitutive expression by epidermal keratinocytes restricted to large, postmitotic cells. Keratinocyte cultures consisting of strain N (epidermal, *A,B*), OKG4 (gingival, *C,D*), OKF4 (floor of mouth, *E,F*), and OKP7 (soft palate, *G,H*) were treated with 0.1% DMSO *(A,C,E,G)* or 10^{-7} M RA *(B,D,F,H)* for the final 2 d before fixation and were immunostained for K19. *Arrowheads, A* indicate rare large, postmitotic cells that were the only $K19^+$ epidermal keratinocytes in cultures growing under control conditions, in contrast to the 10–20% of the small, proliferative cells in *B* that became $K19^+$ after 2 d of exposure to RA. Increasing proportions of small, proliferative $K19^+$ cells were present under control conditions in cultures of parakeratinizing gingival and nonkeratinizing floor-of-mouth and soft palatal cells. These subtypes also were more responsive to RA-induced K19 expression. *Scale bar,* 100 μm.

and involucrin-positive (Watt and Green, 1981; Barrandon and Green, 1985; and our unpublished results), were $K19^+$ in low [RA]. Exposure of epidermal or gingival keratinocytes to 10^{-7} M RA for 2 d resulted in K19 expression by some of the small, proliferative cells **(Fig 3*B,D*)**. After continuous exposure to 10^{-7} M RA for two passages during 2 wk, more than 50% of epidermal keratinocytes had become $K19^+$, yet intracolony heterogeneity persisted (data not shown).

The observed heterogeneity of K19 expression within cultured keratinocyte strains (also exhibited by cells grown in the serum and fibroblast feeder layer system; data not shown) was surprising; it had not been possible to discern this from the earlier protein and mRNA analyses of total culture extracts (Lindberg and Rheinwald, 1990; Crowe *et al*, 1991) or our Western blot analysis **(Fig 1)**. Immunostaining of fixed cultures revealed that the heterogeneity of constitutive and RA-inducible K19 expression is intraclonal **(Fig 3)**. Single cell-derived, clonal epidermal keratinocyte populations were also heterogeneous in their response to RA (data not shown), demonstrating that the cell strains we studied were not mixtures of cells having several intrinsically different K19 expression phenotypes. Thus, all cells in a genetically homogeneous cultured keratinocyte strain are not always equally responsive to RA induction of K19. Instead, they can be in a state either permissive or nonpermissive for K19 expression, and only in the former can they be induced by RA to express K19.

Overexpression of RARβ and RARα in Epidermal Keratinocytes Enhances RA Inducibility of K19 Expression in Conventional Culture To test our hypotheses about the role of RARβ in K19 regulation and keratinocyte differentiation, we used defective retroviral vectors to stably overexpress RARβ or RARα in epidermal keratinocyte strain N cells. RAR expression by the transductants was analyzed by Western blotting **(Fig 4)**. The parent strain and LXSN-transduced controls expressed RARα but no

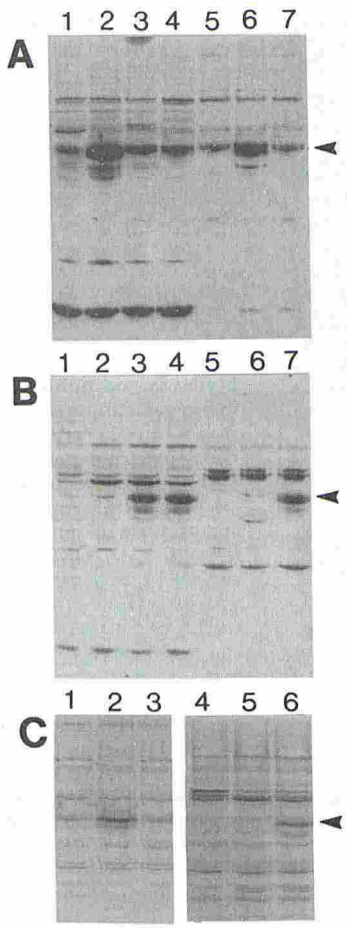

Figure 4. Epidermal keratinocytes can be engineered by retroviral transduction to stably overexpress RARα or RARβ. Stable transductants of strain N epidermal keratinocytes were constructed by infecting with defective retroviruses LXSN (the control, "empty" vector), L(RARα)SN, or L(RARβ)SN, followed by selection for G418 resistance. The transductants were grown in conventional *(A,B)* or organotypic *(C)* conditions. They were then extracted, and the "nuclear" fraction was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunostained with an antipeptide antiserum raised against a unique epitope of either RARα (*A*; *lanes 1,2,3* of *C*) or RARβ (*B*; *lanes 4,5,6* of *C*). *A* and *B* are replicate gels. *Lanes 1,2,3* of each contain extracts of the retroviral packaging cell line PA317 expressing the vectors LXSN *(lane 1)*, L(RARα)SN *(lane 2)*, and L(RARβ) *(lane 3)*. *Lane 4* contains an extract of NIH3T3 cells stably transduced with the L(RARβ)SN vector. *Lanes 5,6,7* contain extracts of stable transductants of strain N: N/LXSN *(lane 5)*, N/RARα *(lane 6)*, and N/RARβ *(lane 7)*. In *C*, *lanes 1,2,3* at left and *lanes 4,5,6* at right are replicate gels of extracts of organotypic cultures of N/LXSN *(lanes 1,4)*, N/RARα *(lanes 2,5)*, and N/RARβ *(lanes 3,6)*. The RARs migrate as ~54-kDa proteins *(arrowheads)*. Despite the cross-reactivity of these antisera with extraneous proteins that were present at similar levels in all transductants of each parent cell line studied, we readily detected elevated levels of RARα in the RARα transductants, above that expressed endogenously by all cell types, and of RARβ in the RARβ transductants.

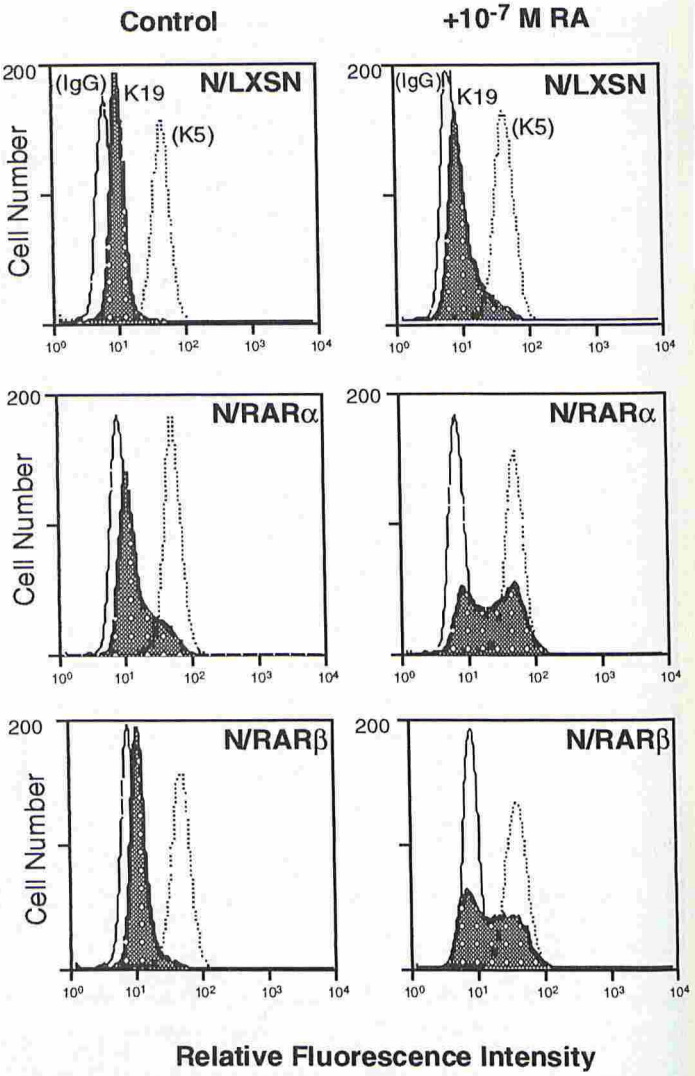

Figure 5. Epidermal keratinocytes that overexpress RARβ or RARα exhibit substantially increased RA inducibility of K19 expression in conventional culture. Cells received 0.1% DMSO as a control *(left)* or 10^{-7} M RA *(right)* for the final 2 d of culture before FACS analysis for K19 content. *Solid outline*, nonimmune mouse IgG; *dotted outline*, K5 antibody; *filled curve*, K19 antibody.

detectable RARβ, consistent with previous mRNA analyses (Crowe *et al*, 1991; Stellmach *et al*, 1991; Vollberg *et al*, 1992). The RARα transductants expressed this receptor type at levels about 5-fold higher than normal, and the RARβ transductants showed a strong signal for RARβ. The normal soft palatal keratinocyte strain OKP7, which in an earlier study was found to express higher levels of RARβ mRNA than any other keratinocyte subtype (Crowe *et al*, 1991), did not express enough RARβ protein to be detected on our Western blots (data not shown). Thus, the level of RARβ expressed by our epidermal keratinocyte transductants substantially exceeded that of any normal nonkeratinizing subtype.

FACS analysis revealed that both the RARβ and the RARα transductants exhibited a substantially increased RA inducibility of K19 expression **(Fig 5)**. After a 2-d exposure to 10^{-7} M RA, ~40-60% of the cells became $K19^+$ in N/RARα and N/RARβ cultures, in contrast to ~5-15% in N/LXSN cultures. The RAR overexpressers, however, remained mostly K19 negative during growth in low [RA], thereby remaining different from normal nonkeratinizing oral cells. These FACS results were confirmed by microscopic examination of immunoperoxidase-stained cultures (data not shown). In the experiment shown in **Fig 5**, the control N/RARα culture had a significant proportion (~15%) of $K19^+$ cells. Microscopic examination of immunoperoxidase-stained cultures disclosed that most of these were large, terminally differentiated cells, which are also present in varying percentages (increasing as cells approach the end of their replicative life span) in control, untransfected epidermal keratinocytes. Neither RARα or RARβ overexpressers appeared to have acquired an increased sensitivity, with respect to K19 induction, to very low RA concentrations (i.e., $<10^{-10}$ M) (data not shown). In such a low RA concentration, RA-receptor complexes do not form efficiently, considering their calculated K_ds (Allenby *et al*, 1993).

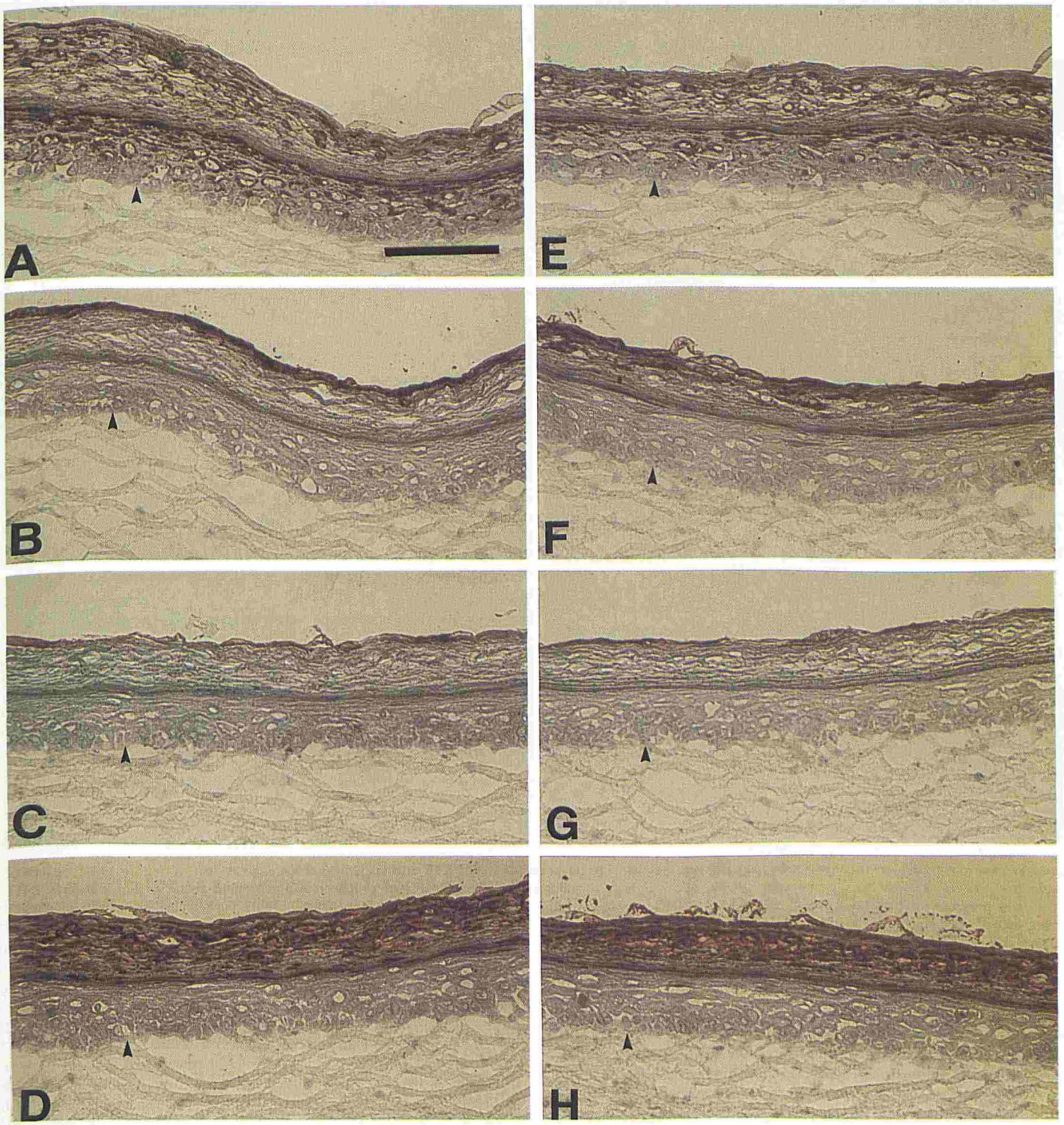

Figure 6. Epidermal keratinocytes form an orthokeratinized epithelium with K19⁻ basal cells in organotypic culture, a differentiation pattern unaltered by high [RA]. Organotypic cultures of epidermal keratinocyte strain N/LXSN were prepared and cultured at the air-liquid interface for 10 d in medium containing a low (3×10^{-10} M) RA *(A,B,C,D)* or high (3×10^{-8} M) RA *(E,F,G,H)* concentration. Cryosections were immunostained with monoclonal antibody to K1/K10 *(A,E)*, K4 *(B,F)*, K19 *(C,G)*, or filaggrin *(D,H)*. *Arrowheads* indicate the junction between the basal epithelial cell layer and the collagen gel. *Scale bar*, 100 μm.

Normal Oral and Epidermal Keratinocytes Exhibit Distinctive, Subtype-Specific Patterns of Differentiation and Basal Cell K19 Expression in Organotypic Culture To establish that we had a suitable *in vitro* system with which to detect and characterize changes in differentiation potential resulting from RAR overexpression, we prepared organotypic cultures of the four normal oral and epidermal keratinocyte subtypes and compared their patterns of basal and suprabasal keratin and filaggrin expression. As described in *Materials and Methods*, keratinocytes were cultured on collagen/dermal fibroblast matrices at the air-liquid interface for 10 d in the presence of low or high [RA]. We used 3×10^{-10} M RA as the low [RA] condition because we found that

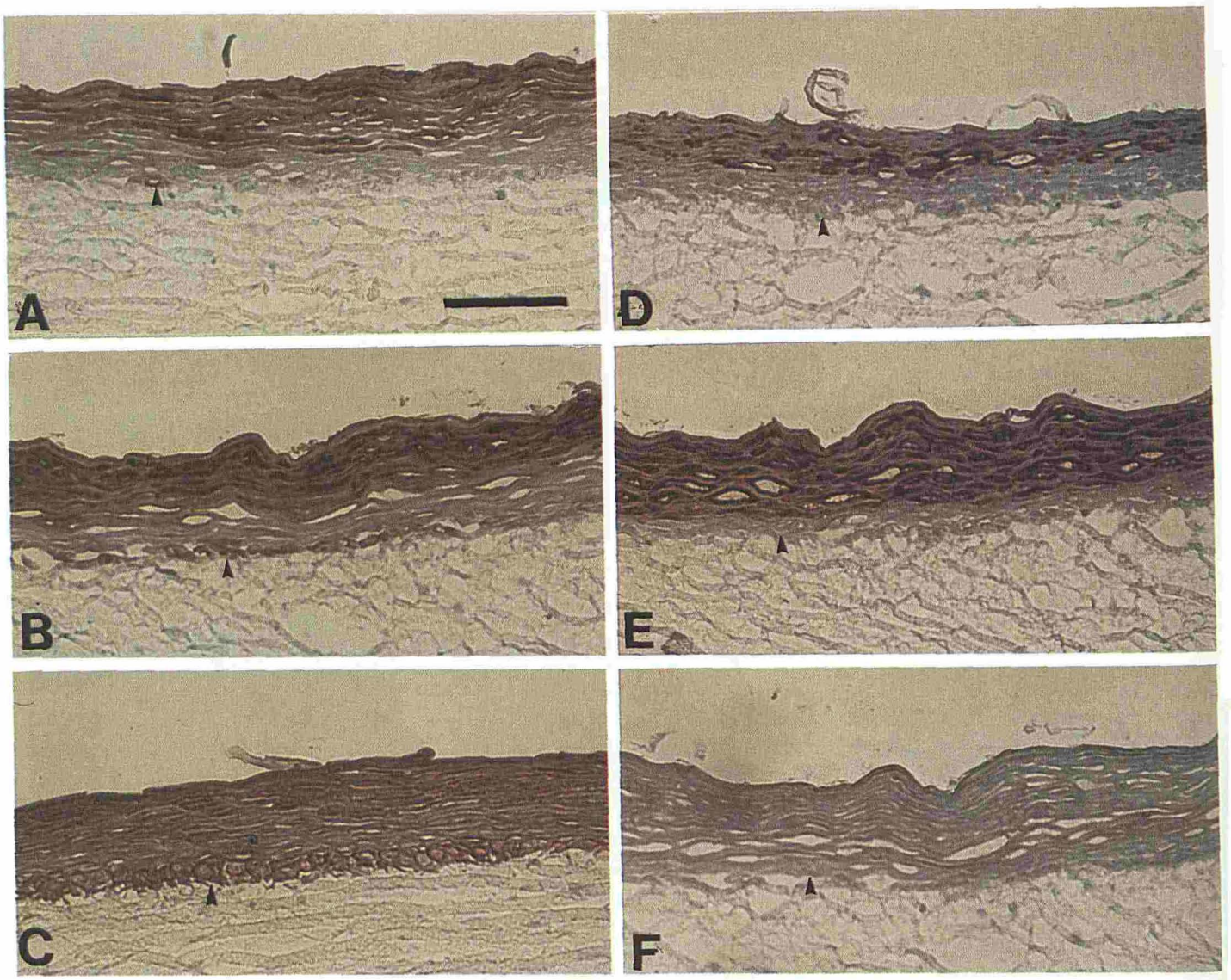

Figure 7. Normal oral keratinocyte subtypes express distinctive patterns of basal K19 and suprabasal K4 expression in organotypic culture, and do not express filaggrin. Organotypic cultures of the indicated oral keratinocyte strains were prepared and incubated at the air-liquid interface for 10 d in the presence of low (3×10^{-10} M) RA. *A*, OKG4 (gingival); *B,D,E,F*, OKF4 (floor of mouth); *C*, OKP7 (soft palate). Cryosections were immunostained with antibodies specific for K19 *(A,B,C)*, K1/K10 *(D)*, K4 *(E)*, or filaggrin *(F)*. *Arrowheads* indicate the junction between the basal epithelial cell layer and the collagen gel. *Scale bar*, 100 μm.

without a low-level RA supplement, the 0.5% bovine serum–containing organotypic culture medium yielded for all subtypes a thin, flattened epithelium, as described previously (Asselineau *et al*, 1989). We chose 3×10^{-8} M as an appropriate high [RA] condition because it is well above the K_d for RA binding to RARs ($\sim 5 \times 10^{-10}$ M) (Allenby *et al*, 1993) and it is also lower than concentrations that begin to be growth inhibitory and to cause severe disorganization of organotypic cultures (Kopan *et al*, 1987; Asselineau *et al*, 1989; Asselineau and Darmon, 1995).

Normal and control LXSN-transduced epidermal keratinocytes stratified and differentiated into basal, spinous, and granular layers and formed an enucleated stratum corneum both in high and low [RA] **(Fig 6)**. K1 and K10 were expressed in most of the suprabasal cells, whereas K4 and K13 were expressed at low levels only in some cells of the outermost layers of the stratum corneum. Filaggrin was expressed at high levels in the granular layer and stratum corneum. K19 was not expressed in the basal cells, but there were occasional $K19^+$ cells in the outermost layer of the stratum corneum, possibly representing rare, postmitotic cells that were already $K19^+$ when the culture was raised to the air-liquid interface.

Normal gingival, floor-of-mouth, and soft palatal keratinocytes cultured under the same organotypic conditions exhibited distinctive, region-specific patterns of basal K19 and suprabasal keratin and filaggrin expression, very different from that expressed by epidermal keratinocytes **(Fig 7, Table I)**. Although all three oral keratinocyte subtypes had substantial numbers of suprabasal $K19^+$ cells, unlike the normal pattern *in vivo* (Lindberg and Rheinwald, 1989, 1990), their basal cell K19 phenotypes were distinctive and mirrored the *in vivo* patterns of the respective subtypes. Gingival keratinocytes formed a parakeratinized epithelium in which the basal layer contained only rare $K19^+$ cells **(Fig 7*A*)**. In the organotypic epithelia formed by floor-of-mouth and soft palatal keratinocytes, K19 was expressed by nearly all of the basal cells **(Fig 7*B,C*)**, and K4 was expressed strongly in all suprabasal cells **(Fig 7*E*)**. None of the oral subtypes expressed filaggrin **(Fig 7*F*)**, in contrast to epidermal keratinocytes. Thus, the organotypic culture system permitted the expression of differentiation programs other

Table I. Differentiation-Related Proteins Expressed by Normal Human Epidermal and Oral Keratinocyte Subtypes in Organotypic Culture[a]

Subtype (Strain)	Layer	K1/K10 (Low/High RA)	K4 (Low/High RA)	K19 (Low/High RA)	Filaggrin (Low/High RA)
Foreskin epidermal (strain N)	Basal	−/−	−/−	−/−	−/−
	Spinous	+,(−)/+,−	−/−,(+)	−/−	−/−
	Cornified	+,(−)/+,−	−,(+)/−,(+)	−,(+t)/+,−	+/+
Gingival (OKG4)	Basal	−/−	−/−	−,(+)/−,+	−/−
	Spinous	+,−/+,−	+/+	−/−,(+)	−/−
	Cornified	+,−/+,−	+/+	−/+	−/−
Floor of mouth (OKF4)	Basal	−/−	−/−	+,−/+	−/−
	Spinous	−,+/−,+	+/+	−/−,+	−/−
	Cornified	+,−/+,−	+/+	+/+	−/−
Soft palate (OKP7)	Basal	−/−	−/−	+/+	−/−
	Spinous	−,+/−,+	+/+	+,−/+,−	−/−
	Cornified	+,−/−,+	+/+	+/+	−/−

[a] Data are summarized from two experiments conducted as described in the legend of **Fig 7**. Results are indicated separately for the single layer of basal cells, the first several suprabasal cell layers (spinous layer), and the outermost multilayer of flattened cells (cornified layer). Protein expression, determined by immunoperoxidase staining, is indicated by + and −. When a region of the organotypic tissue contained both positive and negative cells, it was scored as −,+ to indicate that the majority of the cells were negative but a substantial minority (>10%) were positive; or as +/(−) to indicate that almost all cells were positive, but rare cells (<10%) were negative. "t" indicates that only the topmost layer of cells were stained. Distinctive and informative differences in keratin and filaggrin expression among the subtypes are highlighted by light shading. Note the clear differences in basal layer K19 expression, closely recapitulating the expression patterns of native tissue *in vivo*. Some subtypes express substantial amounts of K19 in the most differentiated, outer cell layers, uncorrelated with their *in vivo* behavior. Also unlike the normal situation *in vivo*, all keratinocyte subtypes co-expressed K1 and K4 suprabasally in organotypic culture. The relative proportions of these differed as expected among the subtypes (e.g., epidermal keratinocytes expressed much greater levels of K1 and lower levels of K4 than did soft-palate keratinocytes).

than orthokeratinization and, therefore, was suitable for assessing the effects of RAR overexpression in the transductants.

RAR Overexpression Does Not Alter Basal Cell K19 Expression or Suprabasal Differentiation of Epidermal Keratinocytes in Organotypic Culture We then examined the differentiation characteristics and the RA sensitivity thereof of the RARα- and RARβ-overexpressing strain N epidermal keratinocytes. Both formed orthokeratinizing epithelia in organotypic culture, exhibiting no detectable differences from control, LXSN-transduced cells with respect to morphologic differentiation or the expression of K19, K1, K4, or filaggrin, even in the presence of high [RA] (**Fig 8**; compare with **Fig 7*E–H***). The absence of RA-induced K19 expression was especially surprising, in contrast to the enhanced inducibility of these transductants in conventional culture **(Fig 5)**. This was not due to a failure of the cells to overexpress their respective retinoid receptor type in organotypic culture, as shown by Western blot analysis of organotypic culture extracts **(Fig 4C)**. Nor did it result from an inability of RA to reach the organotypic epithelium in biologically significant concentrations, as the expression of K19 and K4 was increased in floor-of-mouth keratinocytes cultured in high [RA] **(Table I)**. In one experiment, the RAR transductants were cultured in the presence of high [RA] beginning 3 d before they were seeded onto the collagen gels, continuing through 4 d of growth to confluence on the collagen gels while submerged in medium, and also during the 10-d incubation period at the air-liquid interface. Cells that had undergone this long-term RA exposure still formed an orthokeratinized epithelium with no basal layer K19 expression, although there was an increase in $K19^+$ cells in the outermost, superficial cell layer (data not shown). Thus, cells that were already $K19^+$ before being influenced by the organotypic culture conditions selectively sorted upward, out of the basal compartment.

The Histogenetic Potential of Gingival Keratinocytes Also Is Unaffected by RAR Overexpression To determine whether RARβ overexpression could convert a keratinocyte subtype more closely related to oral lining mucosal cells toward a nonkeratinizing phenotype, we generated RAR transductants of the normal gingival keratinocyte strain OKG4. In conventional culture, RARβ- and RARα-overexpressing OKG4 cells were more responsive than control, LXSN-transduced OKG4 cells to RA induction of K19. The RAR transductants, however, did not exhibit increased K19 expression or altered suprabasal protein expression in organotypic culture (data not shown). Thus, the responses of gingival and epidermal keratinocytes to RAR overexpression were identical, in that their respective differentiation programs were not redirected toward nonkeratinization.

DISCUSSION

Our characterization of RAR-overexpressing keratinocytes in conventional and organotypic culture provided a robust test of the hypotheses we proposed about the role of RARβ in regulating K19 expression and specifying alternate keratinocyte differentiation programs. The epidermal and gingival keratinocyte transductants expressed substantially higher levels of the RARα and RARβ receptors than any normal, untransduced keratinocyte subtype, including nonkeratinizing oral cells, and they exhibited an enhanced RA inducibility of K19 expression in conventional culture, indicating that the receptor proteins encoded by the vectors were functional. Thus, if the levels of either RARα or RARβ were limiting for a "nonkeratinization" pattern of gene regulation, the transductants should have exhibited an altered phenotype.

Immunomicroscopic and FACS analyses revealed that, in conventional culture, individual cells of all keratinocyte subtypes can exist in either a $K19^+$ or a $K19^-$ state. Nonkeratinizing differentiation potential *in vivo* correlates with a high proportion of $K19^+$ cells, rather than with a high modal K19 content per cell, in conventional culture. Epidermal and gingival keratinocytes that respond to RA attain the same maximum K19 content as soft palatal keratinocytes. Thus, both the constitutive and RA-inducible mechanisms that regulate K19 apparently activate expression only until a cell accumulates a certain maximum K19 content. The heterogeneous RA response of epidermal and gingival keratinocytes suggests that these subtypes can exist in alternative states, either permissive or nonpermissive for RA inducibility, in contrast to floor-of-mouth and soft palatal keratinocytes, which either express K19 constitutively or are uniformly inducible by RA. RARα or RARβ overexpression in epidermal or gingival keratinocytes results in an increased proportion of small, proliferative cells becoming $K19^+$ in response to high [RA] in conventional culture. These RAR overexpressers remain K19 negative when cultured in low [RA], however, so they have not acquired the K19 regulatory mechanism of normal oral nonkeratinizing subtypes.

In organotypic culture with dermal fibroblasts in the underlying collagen matrix, normal floor-of-mouth and soft palatal keratinocytes formed nonkeratinizing epithelia, gingival keratinocytes formed a parakeratinizing epithelium, and epidermal keratinocytes formed an orthokeratinizing epithelium. Increasing the [RA] to

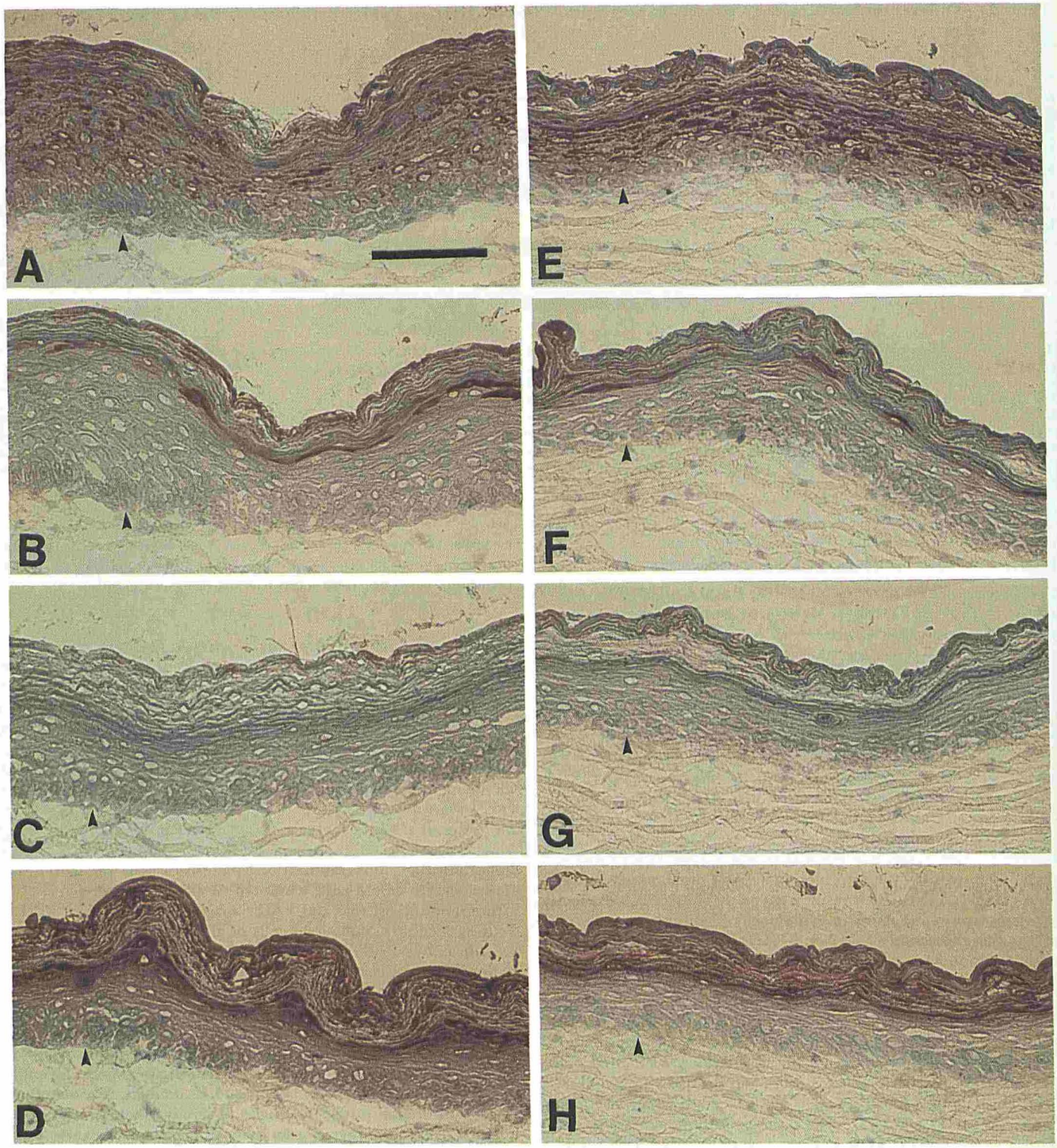

Figure 8. RAR-overexpressing epidermal keratinocytes do not exhibit an RA-inducible alteration toward a nonkeratinizing pattern of differentiation in organotypic culture. Organotypic cultures of N/RARα *(A,B,C,D)* and N/RARβ *(E,F,G,H)* were prepared and immunostained as described in **Fig 6**, except that all cultures received high (3×10^{-8} M) RA during the final 10 d at the air-liquid interface. Antibodies included K1/K10 *(A,E)*, K4 *(B,F)*, K19 *(C,G)*, and filaggrin *(D,H)*. *Arrowheads* indicate the junction between the basal epithelial cell layer and the collagen gel. *Scale bar*, 100 μm.

two orders of magnitude higher than the binding constant for retinoid receptors (Allenby *et al*, 1993) did not induce any subtype to form even an approximate phenocopy of a normal, less keratinized epithelium. Connective tissue fibroblasts that underlie these epithelia *in vivo* and in the organotypic culture system also express RARα and RARβ (for example, see Crowe *et al*, 1991), and their pattern of gene expression is subject to regulation by RA. The differentiation exhibited by each keratinocyte subtype, however, when cultured organotypically with a common dermal fibroblast cell strain, whether in high or low [RA], was distinctive and subtype appropriate. This result is consistent with our earlier xenograft analysis of oral and epidermal keratinocyte differentiation (Lindberg and Rheinwald, 1990) and strengthens the evidence for the primary role of a fixed, intrinsic regulatory program in each

keratinocyte subtype that determines its pattern of differentiation *in vivo*. It seems unlikely that possible differential expression of cellular retinoic acid binding proteins (CRABPs) by different types of stratified squamous epithelia (see Siegenthaler *et al*, 1987) is responsible for the variety of suprabasal differentiation programs exhibited by oral and epidermal keratinocytes or for their sensitivity to RA, considering the absence of epithelial defects in double null CRABPI/CRABPII "knockout" mice (Lampron *et al*,1995).

Our data indicate more limited potential roles for regional fibroblasts and local RA concentration in directing or modulating epithelial differentiation programs than have been proposed in several recent reports. Kautsky *et al* (1995) reported improved differentiation and enhanced resistance to the disorganizing effects of high [RA] by gingival keratinocytes in organotypic culture when a strain of gingival fibroblasts, instead of several other fibroblast strains tested, was used in the underlying collagen gel. We obtained good gingival differentiation using dermal fibroblasts in the collagen gel, however, which suggests the likelihood of substantial variability among fibroblast strains, unrelated to their site of origin, in promoting optimal growth and tissue formation in organotypic culture. On the other hand, the patterns of suprabasal K1 *versus* K4 expression of the four keratinocyte subtypes we examined did not recapitulate precisely the respective normal *in vivo* phenotypes, indicating that further study is required to identify all the factors necessary to permit or promote completely normal histogenesis in organotypic culture. Asselineau and Darmon (1995) reported suppression of K1 and an increase in suprabasal K4 and K19 in the severely disorganized, metaplastic organotypic epithelium formed by epidermal keratinocytes exposed to very high (10^{-6} M) RA. They interpreted this result as indicating that epidermal keratinocytes can be converted to a completely normal oral, nonkeratinizing cell type simply by adjusting the [RA] in their environment. In the abnormal epithelium formed by epidermal keratinocytes in that study, however, K19 was not expressed in the basal cells, indicating clearly that a normal, nonkeratinizing differentiation program had not been activated.

For the purposes of this study, the distinctive patterns of histogenesis expressed by the three oral subtypes demonstrated that the organotypic culture system we used permitted the expression of differentiation programs other than orthokeratinization and, therefore, was suitable for detecting altered differentiation resulting from RAR overexpression. Based on the RA inducibility of K19 in keratinocytes growing in conventional culture, one might have predicted that in organotypic culture, RA would induce K19 expression in the basal cells of the epidermal and gingival RAR transductants and would also alter their suprabasal differentiation program toward nonkeratinization. The transductants formed epithelia indistinguishable from those formed by the respective normal subtypes, however, including suprabasal K1/K10 and (for the epidermal transductants) filaggrin expression, as well as complete absence of constitutive or RA-inducible K19 expression in the basal cells. Thus, the levels of RARα and RARβ can influence retinoid inducibility of K19 in keratinocytes only in certain limited circumstances, which include conventional culture but not organotypic culture, or, presumably, the epithelial tissue *in vivo*.

Basal keratinocytes apparently undergo a change from RA inducibility to RA insensitivity for K19 regulation when they are transferred from conventional to organotypic culture. The mechanism responsible for this conversion to a state resembling the *in vivo* situation, in which the basal keratinocytes of the epidermis do not express K19 in response to RA, remains to be determined. There are several important differences between conventional and organotypic culture (see Bilbo *et al*, 1993). These include a morphologic change of the basal cells to a more cuboidal shape, a basolateral/apical membrane polarization and distribution of surface integrins, formation and adhesion to a basal lamina, and entrance into a very slowly dividing or quiescent proliferative state. Identification of the extracellular signals and the intracellular mediators that switch keratinocytes between RA-responsive and RA-unresponsive states should be accessible by manipulating culture conditions.

One of our hypotheses was that RARβ plays a special role in regulating K19 expression in keratinocytes. The experiments showed, however, that an elevated level of RARα was equally effective in enhancing RA-inducible K19 expression in conventional culture. The absence of apparent defects in the stratified squamous epithelia of RARα-, RARβ-, or even RARγ-deficient transgenic mice (reviewed by Kastner *et al*, 1995) indicates considerable redundancy of function for the RAR receptor types, at least in the mouse. Two transgenic mouse strains expressing dominant-negative mutant RARs (which interfere with the function of all RAR receptor types) targeted to stratified squamous epithelia, however, exhibited moderate to severe functional and histologic defects in the epidermis, ranging from loss of barrier function but otherwise normal histology and gene expression (Imikado *et al*, 1994) to a very thin, atrophic epithelium (Saitou *et al*, 1995). General retinoid receptor function, therefore, seems essential for epithelial viability or for regulating a subset of differentiation-related genes, despite the current obscurity about the functions of individual members of the RAR family.

Our study demonstrates that RARβ alone does not specify the expression of alternate sets of genes that define different stratified squamous epithelial differentiation programs. Thus, RARβ cannot be placed in the small family of "master" differentiation control genes identified so far, such as myoD for skeletal muscle (Davis *et al*, 1987) and PPARγ for adipose tissue (Tontonoz *et al*, 1994). Furthermore, our experiments clearly indicate that basal cell K19 and suprabasal differentiation-related protein expression in stratified epithelia are subject to regulation by an RA- and RAR-independent mechanism. An avenue to discovering this mechanism may be the study of squamous cell carcinomas and premalignant oral epithelial cells, many of which are natural mutants of K19 regulation that either express K19 inappropriately or are insensitive to induction of K19 (Wu and Rheinwald, 1981; Wu *et al*, 1982; Lindberg and Rheinwald, 1989, 1990; Hu *et al*, 1991).

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