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The Role of Retinoic Acid Receptors in
Oral Epithelial Differentiation

by

Mikael B. Kautsky

A dissertation submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy
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to Offer Degree Department of Oral Biology

Date May 30, 1997

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University of Washington

Abstract

The Role of Retinoic Acid Receptors
in Oral Epithelial Differentiation

by Mikael B. Kautsky

Chairperson of the Supervisory Committee
Professor Beverly A. Dale
Department of Oral Biology

Retinoic acid (RA) is important for regulation of epithelial differentiation. RA exerts its effects via nuclear retinoic acid receptors (RARs), which act as transcription factors for numerous genes. Three RAR subtypes have, sofar, been identified and research suggests that each subtype has specific functions in the cell. Within the oral cavity epithelial differentiation exhibits regional variation from the noncornified lining mucosa to the cornified masticatory mucosa. It was hypothesized, that RA influences oral epithelial differentiation and that this regulation is mediated by a region specific expression of the RARs. The goal was to investigate the effects of RA on oral epithelial differentiation and the role of retinoic acid receptor (RARs) in this process by finding an association between specific patterns of RAR expression and specific patterns of oral epithelial differentiation. Oral epithelial cells were grown in an organotypic culture system in various RA concentrations and with different types of fibroblasts. Keratinocyte differentiation was analyzed by histology and immunohistochemistry and/or immunoblotting, and expression of the RARs was detected by RT-PCR experiments. Using this approach, it was shown that RA exerts major regulatory effects on oral epithelial differentiation by inhibiting epithelial cornification. This regulation is modified by the underlying fibroblasts. Examination of the RAR expression in the cultured epithelia revealed that RARβ expression is inversely associated with the degree of epithelial cornification. In contrast, RARα and RARγ expression do not vary

appreciably with a change in epithelial differentiation *in vitro*. Similar RAR expression patterns were found *in situ* when comparing oral epithelia from a cornified and a noncornified region. These results are consistent with the hypothesis that RARβ plays a major role in the RA-sensitive regulation of oral epithelial differentiation. However, preliminary experiments with a RARβ specific agonist indicated that the increase in RARβ expression may be a secondary phenomenon as a result of the switch from a cornified to a noncornified pattern of oral epithelial differentiation. Continued use of the organotypic culture model and the new approach of using RAR subtype specific retinoids will shed new light on the functions of RARs in oral epithelia.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABC	=	avidin-biotin-peroxidase complex
AF	=	activation function
C	=	cornified
CAT	=	chloramphenicol acetyltransferase
cDNA	=	complementary deoxyribonucleic acid
CRABP	=	cellular retinoic acid binding protein
CRBP	=	cellular retinol binding protein
dATP	=	2'-deoxy-adenosine-5'-triphosphate
DEPC	=	diethyl pyrocarbonate
DMEM	=	Dulbecco's modified eagle medium
DNA	=	deoxyribonucleic acid
EGF	=	epidermal growth factor
FBS	=	fetal bovine serum
FG	=	filaggrin
GM10	=	a human embryonal dermal fibroblast line
G3PDH	=	glyceraldehyde-3-phosphate dehydrogenase
H&E	=	hematoxylin and eosin
HBS	=	hepes buffered saline
HOFs	=	human oral fibroblasts (usually followed by a number denoting the tissue from which the fibroblasts originated)
HOKs	=	human oral keratinocytes (usually followed by a number denoting the tissue from which the keratinocytes originated)
J.G.	=	a human adult dermal fibroblast line
K	=	keratin (usually followed by a number denoting its classification according to Moll's catalog)
KBM	=	keratinocyte basal medium

kDA	=	kiloDalton
MEM	=	Eagle's minimum essential medium
mq	=	Millipore super filtered
mRNA	=	messenger ribonucleic acid
N	=	noncornified
PAGE	=	polyacrylamide gel electrophoresis
PCR	=	polymerase chain reaction
PO	=	acidic ribosomal phosphoprotein (also abbreviated as RPO)
PPAR	=	peroxisome proliferator-activated receptor
proFG	=	profilaggrin
RA	=	all *trans*-retinoic acid
RAR(s)	=	retinoic acid receptor(s)
RARE(s)	=	retinoic acid response element(s)
RE(s)	=	response element(s)
RNA	=	ribonucleic acid
RT	=	reverse transcription
RXR(s)	=	retinoid X receptor(s)
SDS	=	sodium dodecyl sulfate
TGFβ	=	transforming growth factor beta
TR(s)	=	thyroid hormone receptor(s)
Tris	=	tris[hydroxymethyl]aminomethane
TTM	=	tissue transport medium
T3	=	triiodothyronine
VAD	=	vitamin A deficiency
VDR	=	vitamin D receptor

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DEDICATION

To Lydia and Myles with love

CHAPTER I

Background, Hypothesis and Aims

1. Introduction:

It has been known for almost seventy years, that vitamin A is an important growth factor. The active forms of vitamin A in the body exist as retinol, retinal and retinoic acid. Retinol is the transported form in the body, secreted into the blood circulation by liver in a complex with serum retinol binding protein (SRBP). Retinol is subsequently taken up by target cells where it is metabolized to retinal and/or retinoic acid (RA). The aldehyde form functions as the active mediator for vision (Saari, 1994, review), while RA controls epithelial cell differentiation (Lotan, 1980, review). Moreover, RA is able to trigger the differentiation of embryonal carcinoma cells (Strickland and Mahdavi, 1978), leukemic HL60 cells (Breitman et al, 1980), and to induce striking digit pattern duplications when locally applied to the developing chick limb bud (Tickle et al, 1985). RA is also able to exert pleiotrophic effects on odontogenesis when applied before the formation of dental lamina (Kronmiller et al, 1995) and it forms a concentration gradient across the limb (Thaller and Eichele, 1987) and across the mandible (Kronmiller et al, 1995), confirming the importance of this retinoid as a morphogen and suggesting its involvement in early embryonic development, morphogenesis and cell differentiation. This chapter serves to review the effects of RA on epithelial differentiation.

2. Oral epithelial differentiation:

The surface of the human body and the surface of body cavities that communicate with the exterior, such as the oral cavity, are in general covered by a

stratified squamous epithelium. Such epithelia consist of several layers of cells, called keratinocytes, which are tightly attached to one another and so form a protective barrier between the external environment and the underlying connective tissues. Integrity of this barrier is maintained by a system of continuous cell renewal in which keratinocytes in the deepest layers migrate to the surface to replace those that are lost by desquamation. As the individual keratinocyte migrates through the cell layers, it undergoes a series of morphological and biochemical changes that are distinctive for each type of epithelium.

The oral cavity is covered by morphologically distinct types of epithelia in various regions. These epithelial types may be viewed as an adaptation to functional demands of the various regions. Two predominant epithelia in the oral cavity are the cornified epithelium of the masticatory mucosa which covers the hard palate and the gingival tissues surrounding the teeth, and the noncornified epithelium of the lining mucosa which covers most of the rest of the oral cavity (Figure 1.1). A few areas are covered by a third major epithelial type which is morphologically and functionally specialized. Both the cornified and noncornified oral epithelia consist of a basal and a prickle cell layer which are grossly similar in the two epithelia (Squier et al, 1976). However, the higher cell layers exhibit structural differences between the two epithelial types. The uppermost cells in the noncornified epithelia belong to the superficial cell layer. Transition between the prickle cell layer and the superficial layer brings relatively small changes in keratinocyte morphology. The cells become somewhat flatter and loose some of their organelles. However, they remain hydrated and retain relatively normal nuclei. In contrast, in the cornified epithelium, the two uppermost layers are the granular and the keratinized layers. The epithelial cells undergo marked morphologic changes as they move through these two layers (Chen and Squier, 1984). The first change is the appearance of so called keratohyalin granules in the cytoplasm of these cells. Subsequently, these granules and all organelles disappear and the keratinocytes become extremely flattened and dehydrated. This type of cornified epithelium is called orthokeratinized. A variant of this epithelium exists in the oral cavity and is called

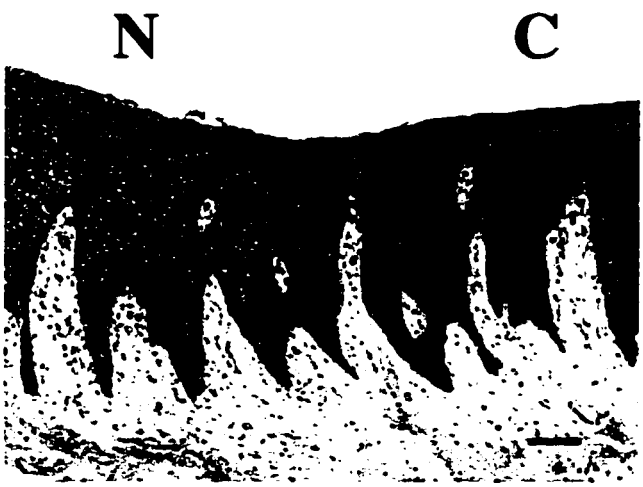

Figure 1.1: Morphology of adjacent noncornified (N) and cornified (C) oral epithelia. A thin section of an oral mucosal biopsy was stained with Hematoxylin and Eosin. Bar is 100μm.

parakeratinized. In this instance, some of the cells retain their nuclei, but because of the dehydration, these nuclei take on a pycnotic appearance.

The morphological changes evident in the differentiation process of the keratinocytes as well as the morphological differences between the two oral epithelial types are accompanied by distinct biochemical characteristics of the cells. Each stage of differentiation can be distinguished by expression of certain structural proteins, called keratins, and by a number of keratin-associated proteins (Roop et al, 1991) (Figure 1.2). Both oral epithelia express keratins 5 and 14 (K5 and K14) in the basal cell layers (Sawaf et al, 1991). In addition, the nonkeratinized epithelium contains K19 in almost all basal cells. In the suprabasal cell layers of the noncornified oral epithelia, two keratins are prevalent, namely K4 and K13. This is in contrast to the expression of the epidermal keratins K1 and K10 in the suprabasal layers of the oral cornified epithelia. A minor component of the cytoskeleton of the cornified oral keratinocytes is K2p (Collin et al, 1992) and the keratin pair, K6 and K16. In addition, a distinguishing feature of the cornified oral epithelia is the expression of the keratin-associated protein, profilaggrin, although a weak expression of this protein may sometimes be found in the noncornified epithelia as well (Reibel et al, 1989). These and other (the above description is by no means exhaustive) biochemical distinguishing features of the different epithelia are commonly employed as useful research tools in the study of epithelial differentiation processes.

3. Retinoic acid effects on epithelial cells and epithelial differentiation:

Vitamin A exerts profound effects on epithelia; in a classic *in vivo* study, Wolbach and Howe (1925) showed that a deficiency leads to squamous metaplasia, while a classic *in vitro* study by Fell and Mellanby (1953) using chick ectoderm explants, showed that a hypervitaminosis causes mucous metaplasia (Lotan, 1980; review). Because of the pleiotropic effects of vitamin A and its pronounced systemic toxicity at

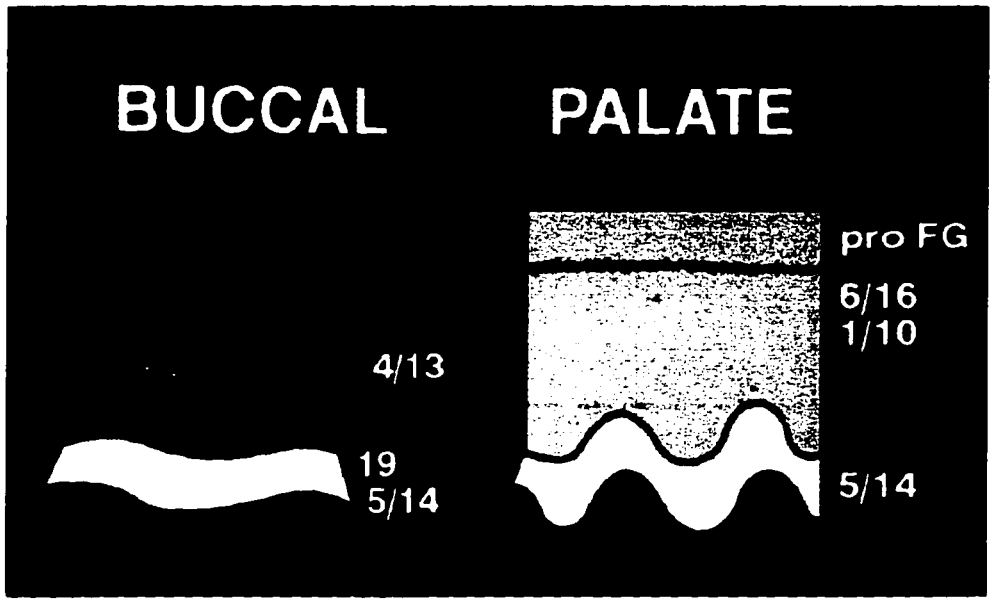

Figure 1.2: Schematic figure of selected markers of differentiation in noncornified (buccal) and cornified (palate) oral epithelia. The epithelia are divided into basal (yellow) and suprabasal (blue and pink) compartments. In the cornified epithelium, the granular cell layer is shown as a black band. The numbers denote expressed keratins according to Moll's catalog (Moll et al, 1982). (Adapted from a slide generously provided by Dr. B. Dale).

high doses, its effects on epithelial differentiation are difficult to interpret in *in vivo* studies. However, the above observations have been substantiated in several studies using conventional cell culture techniques. Excess RA in the growth medium has been shown to alter keratin synthesis and to inhibit formation of cross linked envelopes of epidermal cells (Eichner, 1986). Fuchs and Green (1981) showed in epidermal cell culture that retinoids contained in the fetal calf serum supplement are sufficient to inhibit synthesis of keratins K1 and K10. These two keratins are normally expressed in the suprabasal layer of cornified epithelia such as the epidermis or the gingival and palatal epithelia in the oral cavity (see above). Another important differentiation marker of the cornified epithelia is the keratin associated protein profilaggrin. A dramatic downregulation of profilaggrin by the influence of RA has also been observed in culture (Hohl et al, 1991). These studies, using conventional culture techniques, indicate that RA is a potent inhibitor of terminal differentiation of epidermal cells. Kurlandsky et al (1994) have shown that all-*trans*-RA is the biologically active retinoid in human keratinocytes.

The development of more sophisticated culture systems, e.g. the dermal equivalent (Asselineau and Prunieras, 1984; Asselineau et al, 1987), has provided more physiological *in vitro* growth conditions for the epithelial cells. This technique provides the epithelial cells with a biological matrix on which to attach. It further allows the epithelial cells to get nutrients from the direction of the basal cell layer and to have a surface in contact with the atmosphere. A "skin organ" grown under these conditions allows for better comparison between the *in vivo* and the *in vitro* differentiation programs of the cells. Thus, when human epidermal cells are cultured on floating rafts of collagen and fibroblasts (the dermal equivalent), they stratify in an *in vivo*-like fashion at the air-liquid interface. Addition of RA to the medium under such conditions not only suppresses the expression of the suprabasal keratins K1 and K10 and other markers of cornified epithelia, such as profilaggrin, but also induces synthesis of keratins associated with noncornified epithelia, e.g. the K4 and K13 pair, K7 and K19 (Kopan et al, 1987;

Asselineau et al, 1989; Asselineau and Darmon, 1995). These observations suggest that RA is not only a potent suppressor of the terminal differentiation exhibited by cornified epithelia, but actually functions as a regulator in the choice of differentiation pathways of the epithelial cell.

Most studies concerned with the effect of RA on epithelial differentiation have been carried out on skin epidermis. Much less is known about its effects on oral mucosa. However, it is clear that oral epithelial cells, indeed, are responsive to retinoids. Thus, in cultures of rat oral epithelial cells, enhancement of keratin K13 by retinoids was observed by Buckley and Middleton (1987). Furthermore, in the course of the present project, it has been shown that RA has the same effect on human oral keratinocytes in culture (Kautsky et al, 1995). It has been established in biopsy tissue that while buccal oral epithelium, which is noncornified, expresses K13, cornified epithelium from hard palate does not (Ouhayoun et al, 1985; Clausen et al, 1986). Conversely, it has been shown that the epithelium of masticatory mucosa expresses keratins K1 and K10, but that these are absent in the epithelium of the lining oral mucosa. It is especially notable that the keratins expressed in noncornified oral epithelia (K13 and K19) are upregulated by RA, while those expressed in cornified oral epithelia (K1 and the keratin-associated protein, profilaggrin) are downregulated. Thus, these observations extend the results from the studies on epidermal keratinocytes and are consistent with the hypothesis that RA plays an important regulatory role in the differentiation process of oral epithelial cells as well.

However, RA is most likely not the sole regulator of terminal keratinocyte differentiation and the process of entering and maintaining the differentiated state is probably mediated by a cascade of biochemical events. Jetten et al (1989) suggested that normal human epidermal keratinocytes in culture terminally differentiate through a program which consists of a retinoid-insensitive step (commitment to terminal differentiation) and a retinoid-sensitive step (expression of the squamous differentiated phenotype). The autocrine growth hormone, TGFβ, may take part in this regulation

(Fuchs, 1990) and extracellular calcium ion concentration seems to be important as well (Watt, 1989). Connective tissue is another important determinant for epithelial differentiation (Briggaman, 1982). Studies by recombination of oral and dermal tissues have shown that the connective tissues are able to influence differentiation and proliferation of keratinocytes (Billingham and Silvers, 1967; Hill and Mackenzie, 1989; Wilson et al, 1992). However, the molecular mechanisms of epithelial-mesenchymal interactions in the oral cavity are still largely unknown and the role of retinoids not understood. Sanquer et al (1990) showed that dermal fibroblasts modulate the effects of retinoids on epidermal growth. In the course of the present project (Kautsky et al, 1995), it was found that subepithelial fibroblasts exert a modulatory effect on the RA-sensitive differentiation of oral keratinocytes.

4. Mechanisms of the RA activities:

The identification of nuclear receptor proteins for RA (Petkovich et al, 1987; Zelent et al, 1989) has confirmed the hypothesis that RA acts at the level of gene transcription, similarly to steroids and other hormones via interaction with specific nuclear receptors which act as transcription factors (Evans, 1988). To date, three different retinoic acid receptors (RARα, RARβ and RARγ) have been cloned and sequenced (Zelent et al, 1989). A comparison between the mouse RARs and human RARs has revealed that there is greater sequence homology in each subgroup of the RARs across the two species, than between the three different RARs in the same species. This observation strongly suggests that the different RARs have different functions. This hypothesis is further supported by tissue specific distribution of the RARs. Using cDNA probes, northern blots have revealed that RARγ seems to be highly restricted to skin in the adult mouse, whereas RARα and RARβ are expressed in a variety of adult tissues. Furthermore, using similar probes in *in situ* hybridization experiments, Noji et al (1989) has revealed that RAR genes are expressed in keratinizing areas of mouse epidermis.

This further supports the hypothesis that RA and RARs play an important role in the terminal differentiation of epithelia. It is conceivable that region-specific expression of the different RARs and subsequent binding of RA may trigger the expression of specific gene products and lead to region-specific differentiation. Currently, it is not known which factors may control such a tissue specific distribution of the RARs, but it is evident that RA plays at least a partial role in this regulation. It has been shown (de The et al, 1990) that RARβ gene expression is responsive to the level of RA via an autoregulatory mechanism. The RA levels may in turn be regulated by cellular retinoic acid binding proteins (CRABPs). Two subtypes have so far been identified and of these, it is the CRABP-II subtype which is the predominant form in human keratinocytes (Astrom et al, 1991). This cytosolic protein is thought to maintain appropriate intracellular RA concentrations for differential regulation of gene transcription via RA/RAR complexes (Maden et al, 1988; Boylan and Gudas, 1991). However, a specific role for the CRABPs has not yet been found. Indeed, transgenic mice with CRABP-I and CRABP-II double knock-outs were essentially indistinguishable from wild-type litter mates in their development (Lampron et al, 1995). In addition, a second family of retinoid responsive nuclear receptors (RXRs), which preferentially bind the RA metabolite 9-*cis*-RA, has been characterized (Mangelsdorf et al, 1990). RXRs form heterodimers with RARs and function as coregulators of gene transcription (Yu et al, 1991). In addition, RXRs heterodimerize with a number of other nuclear receptors, e.g. the thyroid receptor (TR), the vitamin D receptor (VDR), the peroxisome proliferator-activated receptor (PPAR) and also a host of orphan receptors (Mangelsdorf et al, 1995). The RXR interactions increase the transactivating activity of the affected receptor (Leng et al, 1994; Leng et al, 1995) and therefore, the RXRs may in a sense function as the master regulator of many nuclear receptor dependent signaling pathways. Nevertheless, RARs are the distal link in the all-*trans*-RA mediated regulation of gene transcription. Therefore, one of the goals of this work is to determine if a differential distribution of the RARs correlates to specific patterns of oral epithelial differentiation.

5. The RARs:

RARs belong to the superfamily of the steroid/thyroid nuclear receptors. They were identified by a series of experiments in the 1980's by employment of nonstringent hybridization techniques using first steroid hormone probes and subsequently the newly cloned RAR probes (Petkovich et al, 1987; Brand et al, 1988; Krust et al, 1989). It was further discovered that each of the RAR subtypes are expressed in multiple isoforms as result of alternate splicing and usage of different promoters (Leid et al, 1992). The known isoforms are $\alpha 1$ and $\alpha 2$, $\beta 1$ to $\beta 4$ and $\gamma 1$ and $\gamma 2$. The human RARs have been mapped to chromosome 17, band q21.1 for RARα, chromosome 3, band p24 for RARβ and chromosome 12, band q13 for RARγ (Mattei et al, 1991). The products of the RAR genes can be divided into six regions based on homology and diversity among themselves and homology to other nuclear receptors (Figure 1.3). These regions are named with capital letters in alphabetical order starting with A from the 5' end of the gene product and ending with F at its 3' end. Regions A and F show a relatively low degree of homology between the RAR subtypes (< 25%), while regions C and E are highly homologous in their amino acid sequences (approximately 90%). Regions B and D display moderate amino acid sequence homology between the three subtypes (Leid et al, 1992).

The RARs are in essence ligand activated transcriptional regulators and this is mirrored in the specific functions of the various domains of these gene products. Two independent transcription activation domains, namely the A/B and the D/E regions, have been identified. These are called AF 1 (activation function) and AF 2, respectively and have been shown to exert synergistic activation on transcription under certain conditions (Nagpal et al, 1993). The ligand binding activity is provided by the E region. As

Figure 1.3: A general representation of the transcript of retinoic acid receptors (RARs). The gene product is divided into six regions (A-F) based on homology among the three RAR subtypes. No shading represents homology at the amino acid level of less than 25%, light shading represents moderate homology and dark shading illustrates regions of more than 80% amino acid homology.

mentioned above, this region is highly homologous between the three RARs and is able to bind all-*trans*-RA as well as 9-*cis*-RA with high affinity. In addition, this region serves as stabilizer for the protein-protein interactions between the receptors in their formation of dimers. *In vivo*, the RARs function as heterodimers with the RXRs in their regulatory role of gene transcription (Yu et al, 1991). The final major function necessary for the regulatory activity of the RARs is the recognition and binding of specific DNA sequences called RAREs (retinoic acid response elements). Region C is responsible for this activity. At the protein level, this domain contains two zinc finger structures which are frequently found in other DNA-binding proteins (Green and Chambon, 1988). Again this region is highly homologous between the RARs and therefore the three subtypes recognize, at least in *in vitro* studies, the same RAREs. The response elements are hexameric oligonucleotide sequences arranged as either direct or inverted repeats and separated by a set number of nucleotides. It is this spacing that seems to provide specificity for the binding between the receptor and the response element. The RAR/RXR heterodimers are able to recognize direct repeats of the recognition sequence which are separated by one or five nucleotides (Umesono et al, 1991), while other nuclear receptors recognize the same or similar repeat sequences that are separated by a different number of nucleotides. Finally, the function of the F domain is presently unknown.

6. RAR functions:

The function of RARs has been studied using one of three approaches: By engineering knockout mice via homologous recombination or via introduction of dominant negative receptors into the animal, or by *in vitro* over- and underexpression of the nuclear receptors in transfected cells in cultures and finally, by ligand manipulation. The first two approaches have been used extensively and have led to significant advances in our understanding of the functions of RARs, while the last approach has just started to

be utilized. In order to dissect the functions of specific RARs, subtype specific or even isoform specific ligands are necessary. Although, synthetic ligands with some degree of specificity have now been produced (Charpentier et al, 1995; Beard et al, 1995; Agarwal et al, 1996), most of them still exhibit a certain degree of overlap between the RARs and make unambiguous interpretation of ligand manipulative experiments difficult.

Null mutations of RARα, RARβ and RARγ, as well as isoform-specific knockouts of RARα1, RARβ2/β4 and RARγ2 have been generated (Kastner et al, 1995) The RARβ and the isoform specific RARα1 and RARγ2 deficient mice appear normal (Li et al, 1993; Lohnes et al, 1993; Lufkin et al, 1993; Luo et al, 1995;). In contrast, RARα and RARγ mutants display some of the defects of the postnatal vitamin A deficiency (VAD) syndrome, including poor viability, growth deficiency and male sterility (Lufkin et al, 1993; Lohnes et al, 1993; Lohnes et al, 1994). These results indicate that there is a widespread functional redundancy between the RARs at least at the isoform level, but also at the subtype level as in the case of RARβ. Double knockout mutants have been generated (Lohnes et al, 1994; Mendelsohn et al, 1994; Lohnes et al, 1995) and most of these exhibit a dramatically reduced viability and also recapitulate most of the VAD syndrome malformations. These studies demonstrate that the vitamin A effects on development are indeed mediated via the RARs, but do not show the specific functions of each RAR subtype. Interestingly, CRABPI and CRABPII do not seem to be critically involved in the transduction of the RA signals during development, since CRABPI/CRABPII double mutant mice are essentially normal (Lampron et al, 1995).

Sofar, RAR knockout mice have failed to demonstrate any dramatic effects of RARs on epithelial differentiation, except in the case of the RARγ mutants which exhibit squamous metaplasia of the seminal vesicle and the prostate gland epithelia (Lohnes et al, 1993). An alternative *in vivo* approach, namely the introduction of dominant negative RARs into transgenic mice, has supplied more definitive support for the role of RARs in epithelial formation and differentiation. Saitou et al (1995) targeted a dominant negative

RAR to the mouse epidermis by placing a K14 promoter as the 5' regulatory sequence in the RAR construct. Transgenic mice, which expressed this transgene in the basal cell layer of epidermis, exhibited a dramatic suppression of epidermal maturation. In another experiment employing the same approach, but placing a K1 promoter in front of the dominant negative RAR to achieve expression in the suprabasal cell layers, Imakado et al (1995) found that this disruption of RA signaling severely compromised the functional properties of epidermis, especially with respect to barrier function. The two latter studies seem to implicate the RARs in important formative and maintenance functions in the epidermis. However, it is believed that the dominant negative RAR effect is mediated by a titration of other components necessary for RAR function. Therefore, because of the close interaction of RARs and RXRs, the above findings do not exclude the RXRs as the regulators of epithelial functions. Likewise, many other factors which interact with the RXRs, e.g. the thyroid hormone receptors or the peroxisome proliferator-activated receptor (PPAR) (Kliewer et al, 1992), can not be excluded on the same basis. In addition, the RAR dominant negative experiments do not provide any information about the functional specificity of the RARs at the subtype or even less at the isoform levels.

The second approach, i.e. *in vitro* transfection studies, has been utilized to look at the effect of specific RAR subtypes on selected markers of epithelial differentiation or on CAT-constructs containing RA-responsive promoters (Blumenberg et al, 1992). In this study, it was found that the RARs indeed have profound effects on several keratin promoters, but interestingly, meaningful differences between the three RAR subtypes were not found. The results of these studies further reinforce the functional redundancy of the RARs, but leave the question of their physiological significance unanswered.

7. RAR expression:

Another approach which can provide clues about RAR function, though not definitive proof, is to study specific RAR expression in relation to the function of interest. The differential expression of RARs during development (Dolle et al, 1990) and in adult tissues provides the basis for the hypothesis that the different RAR subtypes (and isoforms) are responsible for specific cellular functions. In the rat, RARα is widely expressed in all organs examined (Wan et al, 1992), while RARβ expression is absent in the intestine and the spleen, but present in other internal organs. RARγ is expressed in the reproductive systems and is the major RAR subtype in epidermal tissues. In regard to epithelial expression of RARs several studies have been conducted. Elder et al (1991) found that RARγ is the predominant RAR subtype in human adult keratome biopsies, while the RARα transcript is detectable at lower levels and RARβ not at all. In addition, RAR expression was not affected by topical application of RA-containing cream prior to the biopsies. In contrast, cultured dermal fibroblasts responded to RA treatment with an increase in RARβ expression. The above results were largely corroborated by Redfern and Todd (1992). In contrast, Crowe et al (1991) reported that RARβ expression was induced in response to RA treatment in cultured keratinocytes of several types, both epidermal and oral. The RARβ expression was further correlated with keratin 19 expression and therefore, possibly with the differentiation state of the cells. Darwiche at al (1994) analyzed RAR (and RXR) expression in mouse cervical epithelia. In this study, which involved a comparison between a stratified squamous and a simple columnar epithelia, RARα was expressed in both epithelia, while the RARγ transcript was associated with the stratified squamous epithelium only and RARβ with the simple columnar epithelium only. The two latter studies indicate that RARβ and possibly RARγ are involved in the RA sensitive modulation of epithelial differentiation.

8. Hypothesis and aims of the study:

As discussed above there are numerous indications in the literature that each RAR subtype fills specific physiologic functions. It was, therefore, hypothesized that the regional variation in oral epithelial differentiation is due to a differential expression of the RARs.

The goal of the present study was to substantiate this hypothesis by experimentally manipulating oral epithelial differentiation and correlating such differentiation to RAR expression. Oral epithelia were chosen as a model for this purpose, since at least two contrasting patterns of epithelial differentiation, the cornified masticatory epithelium and the noncornified lining epithelium, exist in close proximity in the oral cavity. Thus, the objective was to be able to substantiate any experimental findings *in vitro* with the actual *in situ* RAR expression in two divergent epithelial tissues.

The project was divided into two main goals: First, to investigate RA effects on oral epithelial differentiation in culture and second, to firmly establish an association between expression of the RAR genes and specific patterns of oral epithelial differentiation. The specific aims of the study were:

1a. To modify and use the dermal equivalent culture model (Asselineau et al, 1987) for growth and differentiation of oral epithelia.

1b. To investigate the effects of all-*trans*-RA on differentiation of oral keratinocytes in culture. It was hoped that manipulation of epithelial differentiation by RA *in vitro* would enhance the natural regional variation of oral epithelia, thus making distinction between various differentiation programs even more evident.

2a. To determine RAR expression in cultured oral epithelial cells at the mRNA level by use of one or more molecular biology techniques.

2b. To separate oral epithelium from underlying connective tissues and to determine the *in situ* distribution of RARs in epithelia with naturally diverging patterns of differentiation.

2c. Finally, to analyze data from the proposed experiments in order to find associations between oral epithelial differentiation and RAR expression.

The ultimate objective of this study was to provide information about the role of RARs in epithelial cell differentiation. Such knowledge would prove useful for development and improvement of management strategies of retinoid responsive diseases involving skin and mucosal differentiation, e.g. psoriasis, leukoplakias and carcinomas.

CHAPTER II

Materials and Methods

1. Introduction:

This chapter describes the basic methodology employed for the various phases of this study. During the eight years that this project has been in progress, development of biomedical sciences has been extremely rapid with new laboratory techniques emerging and old techniques being constantly improved. Therefore, in order to keep up with progress, the laboratory techniques used in this project were constantly modified or changed, be it for the goal of getting better and more easily interpretable results, for time-saving reasons or for financial considerations. In this chapter, the methodology most consistently used throughout the project will be described. Any modifications of or deviations from the basic methodology will be dealt with separately in conjunction with the description of the experiments in the following chapters.

This project is conceptually quite simple. The correlation between oral epithelial differentiation and keratinocyte RAR expression was examined in two parallel systems. The main bulk of work was done using a well-differentiating organotypic culture system (Figure 2.1) and the results from these experiments were then confirmed by examination of *in situ* RAR expression of two different oral epithelia. The experiments thus involved the use of the culture model to derive epithelia of various differentiation patterns or the use of the oral epithelia directly. Total RNA was extracted from the keratinocytes and reverse transcribed. The resulting cDNA was then used as template in semi-quantitative polymerase chain reactions to estimate the level of RARs expressed. The RAR expression was finally correlated to specific differentiation patterns of the epithelial tissues.

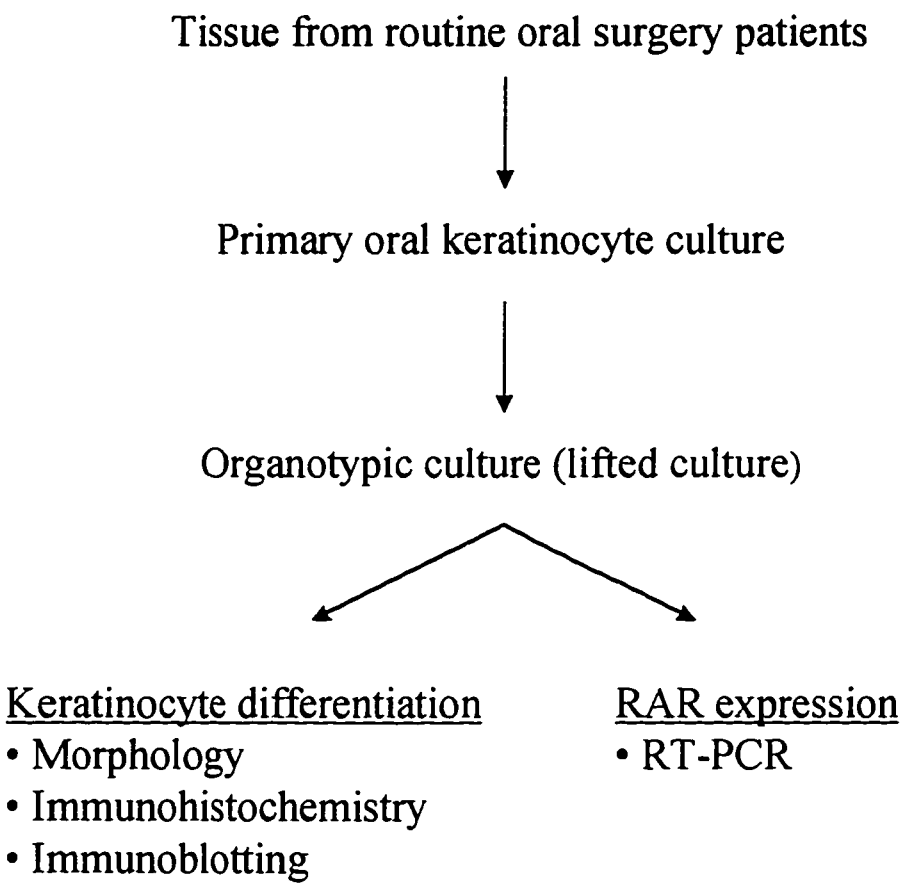

Figure 2.1: Flowchart of the experimental approach for the tissue culture experiments in the present study.

During the course of this work several questions which were not part of the original proposal arose and were pursued to various degrees of resolution. The methods used in these "branching" experiments will not be described in this chapter, but will be mentioned in conjunction with the description of the specific experiments in the following chapters.

2. Cell Culture:

2a. Primary and secondary keratinocyte cultures:

Oral tissue was obtained from patients undergoing routine oral surgical procedures. Epithelial cells were separated from the underlying tissue essentially as described by Oda and Watson (1990). The tissues were placed in ice cold Tissue Transport Medium (TTM) (100mL Hepes Buffered Saline[1], 200µL penicillin/streptomycin and 100µL Fungizone) and transferred to the laboratory. Each tissue was trimmed from excess connective tissue and cut into approximately 2x2mm small pieces. Whenever possible, one of the pieces was withheld for fixation and histologic examination. The other tissue fragments were washed four times in TTM and incubated overnight at 4°C in dispase (6mg/mL HBS, Sigma Chemical Company, St. Louis, MO). The next day, epithelium was mechanically separated from the connective tissue[2], transferred to a 15mL centrifuge tube and incubated in 5mL trypsin-EDTA

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[1] Recipe for HBS:

35.7g	HEPES (Sigma Chemical Co. free acid)
3.6g	Glucose
1.12g	KCl
37.99g	NaCl
0.710g	Na_2HPO_4
5.9mg	Phenol Red

Dissolve reagents in Millipore super filtered water (mq-water). Bring up to 7.4 pH with 1N NaOH. Make up to 5L and sterile filter. Store at 4°C.

[2] Fibroblast cultures: In some instances, the connective tissues were used for production of fibroblast cultures (see text in section 2b). The fibroblast cultures were usually frozen as passage three cells in

(0.05% trypsin, Gibco, Life Technologies, Inc., Grand Island, NY) at 37°C for 10 minutes. The epithelial sheets were then pipetted up and down for 7 minutes at room temperature (RT) and the trypsinization stopped by addition of a culture medium containing 10% fetal bovine serum. Large epithelial pieces were allowed to sediment to the bottom of the tube and the cell suspension transferred to a new tube. The cells were centrifuged for 5 minutes at approximately 200xg in a clinical centrifuge and the pellet resuspended in 5mL KBM (Clonetics Co., San Diego, CA). These primary and subsequent secondary keratinocytes were cultured on plastic in KBM for approximately two and a half weeks. This procedure yielded in general between $3x10^6$ and $6x10^6$ cells.

2b. Organotypic raft cultures:

Organotypic raft cultures (lifted cultures, dermal equivalent cultures) were grown essentially as described by Asselineau and Prunieras (1984) and the technique is schematically shown in Figure 2.2. Briefly, bovine type I collagen (Bioetica, France) was mixed with a suspension based on Eagle's minimum essential medium (Gibco), 10% fetal bovine serum (FBS, Hyclone Laboratories Inc., Logan, UT) and $2x10^5$ fibroblasts per lattice[3]. Fibroblasts tested included GM10 fibroblasts, a cell line from human fetal

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liquid nitrogen. They were then thawed as needed and used in the organotypic cultures at various passage numbers, but not higher than passage 20.

[3] Recipe for one collagen lattice:

3.22mL	MEM 1.76x*
0.63mL	FBS (see above)
0.35mL	0.1N NaOH
0.20mL	DMEM10%** (see page 23)
0.50mL	DMEM10% + $0.2x10^6$ fibroblasts
2.10mL	collagen in 1:1000 (vol:vol) acetic acid

* Recipe for MEM 1.76x:

75mL	sterile mq-water
44µL	Fungizone, 250µg/mL (Gibco)
88µL	Penicillin/Streptomycin, 10,000E/10,000µg/mL (Gibco)
880µL	Sodium pyruvate (Gibco)
5.1mL	7.5% $NaHCO_3$ (Gibco)
17.6mL	MEM 10x (Gibco)

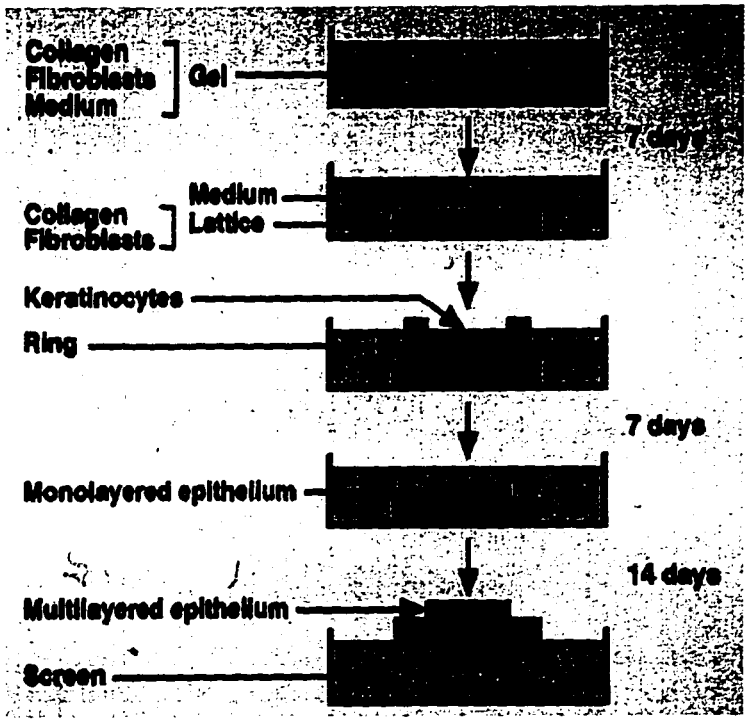

Figure 2.2: Schematic drawing of the organotypic culture methodology. Adapted from Asselineau et al, 1987.

dermal tissue (N.I.G.M.S., Camden, N.J.) and oral and dermal fibroblasts, obtained from appropriate tissues according to conventional explant technique (Cooper and Goldstein, 1973). The collagen lattices were allowed to contract at 37°C for 3-7 days, depending on fibroblast type. Second passage keratinocytes (tertiary) originating from either cornified or noncornified oral mucosa were then seeded on the contracted collagen lattices at a density of $2x10^5$ cells/cm^2 ($3x10^5$ cells/lattice). The cultures were grown submerged in Dulbecco's modified eagle medium (Gibco) for one week, then raised to the air-liquid interface and grown for two more weeks. The medium contained 10% of either normal (control) or delipidized FCS. Delipidization was carried out according to the method of Rothblat et al (1976) and the delipidized product was redissolved at an equivalent protein concentration in Hepes buffered saline. During the first week, all culture media were supplemented with normal FBS, penicillin-streptomycin (Gibco), hydrocortisone (0.4μg/mL) (Gibco), cholera toxin (10^{-10}mol/L) (Calbiochem Behring Diagnostics, La Jolla, CA), insulin (5μg/mL) (Sigma) and transferrin (5μg/mL) (Sigma). At the time of lattice elevation, all media, except the control medium, were switched to delipidized FBS and 1000x stock solutions of all-*trans*-RA in ethyl alcohol (Sigma) were added together with the above supplements under yellow light to a final RA concentration of 0, 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7}mol/L. Cultures were kept in the dark and media changed twice per week. Control cultures received vehicle only. Retinoid concentration in control serum was $5x10^{-7}$mol/L retinol as determined by the Clinical Nutrition Research Laboratory at the University of Washington using the technique of Bieri et al (1979). Retinol was not detectable in the delipidized serum by this technique.

** Recipe for DMEM10%:

90mL	DMEM, low glucose (Dulbecco's Modified Eagle Medium, Gibco)
10mL	FBS
1mL	Penicillin/Streptomycin

3. Epithelial differentiation analysis:

In most early experiments, two lattices were used for each experimental group. One specimen was fixed in methyl Carnoy's[4], sectioned and used for light microscopy of Hematoxylin and Eosin stained sections and for immunohistochemical analysis by the ABC peroxidase method (Hsu et al, 1981). The other lattice was used for protein analysis using 7.5-12.5% gradient SDS-PAGE and immunoblotting. The cultured epithelium was mechanically separated from the collagen lattice and epithelial proteins extracted by homogenization in Urea/Tris buffer (8mol/L and 0.05mol/L, pH 7.6), as previously described by Dale et al (1985). The extracts were frozen at -70°C until analysis. Discontinuous SDS gels were performed by the technique of Laemmli (1970) and stained with Coomassie brilliant blue. Proteins from duplicate gels were electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH) and protein markers of interest were identified with appropriate antibodies (Table 2.1) employing the ABC peroxidase method.

Since the two methods (immunohistochemistry and immunoblotting) were largely comparable in their ability to determine the differentiation profiles of the epithelia, later experiments relied on analysis of morphology and immunohistochemistry only.

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[4] Recipe for Carnoy's fixative:

10mL	Glacial acetic acid
60mL	Methanol
30mL	Chloroform

Table 2.1: Antibodies used for identification of markers of epithelial differentiation.

Antibody	Marker for	Source
5C10	Keratin K1	Sun T-T[a]
AE8	Keratin K13	Schermer A and Sun T-T,[a] unpublished results
KS19.1	Keratin K19	ICN, Immunobiologicals, Lisle, IL
AKH-1	Profilaggrin/filaggrin	Dale et al, 1987
8959 (blots)	Profilaggrin/filaggrin	Fleckman et al, 1985

[a] These antibodies were gencrously provided by Dr. Sun.

4. Total RNA extraction and DNase treatment:

For the majority of experiments total RNA was extracted using the commercially available TRIzol™ reagent (Gibco BRL, Gaithersburg, MD). The use of this reagent is based on the guanidine isothiocyanate RNA isolation method developed by Chomczynski and Sacchi (1987). TRIzol™ was used for total RNA extraction from both cultured cells and from tissues.

Cultured cells: For experiments relying on RNA extraction from keratinocytes grown in the organotypic culture model, four to six parallel lattices were cultured for each experimental condition. One lattice was fixed in methyl Carnoy's and used for analysis of epithelial differentiation (see above). The other lattices were used for RNA extraction. The epithelium was mechanically removed from the underlying collagen matrix and placed in homogenizers containing TRIzol™. Maximally three epithelial samples were placed in 1mL of the RNA extraction reagent. The epithelial tissues were homogenized and allowed to rest at room temperature for 5 - 10 minutes. To each 1mL of homogenate, 0.2mL chloroform/isoamyl alcohol (24/1) (J.T. Baker, Inc, Phillipsburg, NJ) was added, the tubes mixed and allowed to incubate at room temperature for 2 minutes. The samples were then centrifuged for 10 minutes at approximately 12,000xg at 4°C. The supernatant (the upper phase) was then carefully removed and the solvent (the lower phase) was discarded. The supernatant was then subjected to one more round of extraction, this time using water-saturated phenol and chloroform/isoamyl alcohol as described above. The water used in all the RNA experiments was DEPC (diethyl pyrocarbonate) treated water (5'-> 3', Boulder, CO). Each supernatant from the first round of extraction (approximately 0.55mL) was mixed with 0.5mL of the water-saturated phenol and 0.1mL of the chloroform/isoamyl alcohol mixture. The samples were recentrifuged as above and once more the supernatant withdrawn and saved. This supernatant now contained the extracted RNA.

The RNA samples were subsequently concentrated by alcohol precipitation. An equal volume of isopropyl alcohol (J.T. Baker, Inc, Phillipsburg, NJ) was added to the RNA sample and incubated at -20°C for at least one hour, but often overnight. The RNA was then pelleted by centrifugation for 10 minutes at 12,000xg. The pellet was finally washed once with 75% ethyl alcohol (prepared from 100% ethyl alcohol and DEPC-water) and briefly recentrifuged. After the supernatant was discarded, the RNA pellet was allowed to air-dry at room temperature for 15 minutes and then redissolved in an appropriate volume of DEPC-water (usually 25μL).

In order to remove any traces of contaminating DNA, each RNA sample was treated with DNase. The RNA/DNase mixture[5] was incubated at 37°C for 30 minutes, which was followed by another round of phenol/chloroform extraction to stop and remove the DNase. The DNase-treated RNA was diluted with DEPC-water to a volume of 0.5mL and salt concentration was adjusted with 50μL of 2M NaOAc, pH 4.9. Phenol/chloroform extraction and concentration of RNA by isopropyl alcohol precipitation was done as described above. Finally, the pelleted RNA was resuspended in a small volume of DEPC-water (usually < 15μL). A small aliquot (usually 1μL) was used for estimating the RNA concentration and quality by absorbance reading at 260 and 280nm and a second aliquot was used for quality check by agarose gel electrophoresis. Finally, whenever possible, the RNA samples were adjusted to 1mg/mL with DEPC-water as calculated from the A_{260} readings. The formula used for calculating RNA concentrations is as follows:

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[5] RNA/DNase mixture:
25.0μL RNA solution
4.0μL $MgCl_2$
3.5μL 10x buffer
1.0μL RNasin
2.5μL RNase-free DNase I

(All reagents added to the RNA solution were from Promega Corporation, Madison, WI; the first three reagents from Promega's RT kit).

[RNA] = (A_{260})(0.04mg/mL) x D; where D is the dilution factor of the RNA (usually = 100).

The adjusted RNA samples were stored at -80°C until use.

5. Reverse transcription:

The RNA was transcribed into cDNA using the SUPERSCRIPT™ Preamplification System (Gibco BRL) by following the manufacturers instructions for reverse transcription (RT) with oligo(dT)primers. As a rule, five μg (5μL) total RNA was used per reaction and between one to three reactions per experimental group were done at a time and pooled. Each RNA sample was mixed with 1μL oligo(dT)primer (0.5mg/mL) and 6μL DEPC-treated water (up to 12μL). The mixtures were incubated at 70°C for 10 minutes and then immediately placed on ice for at least one minute. In the meantime, a master mix was prepared sufficient in volume for all plus one reactions. For each reaction the master mix consisted of 2μL 10x PCR buffer, 2μL 25mM $MgCl_2$, 1μL 10mM dNTP mix and 2μL 0.1M DTT. Seven (7) μL of the master mix was added to each reaction, mixed and the samples were incubated at 42°C for 5 minutes. Then, 1μL (200 units) of SuperScript II reverse transcriptase was added and the samples were incubated for another 50 minutes at 42°C. The reactions were terminated by incubation at 70°C for 15 minutes and then briefly chilled on ice. Finally, RNA was removed by addition of 1μL RNase H and a last incubation at 37°C for 15 minutes.

At this time the reactions from the same experimental group were pooled and diluted with equal volume DEPC-treated water. The RTs were then used either immediately in the PCR amplifications or stored at -20°C until further use. Typically between 1μL to 2μL of the RT was used per amplification reaction.

6. PCR primers:

Since oligo(dT)primers were used for the RT reactions, primers for the products to be amplified were chosen from their respective 3' ends. The PCR primers and product lengths are shown in Table 2.2. In addition, the choice of this region for the RAR primers conferred specificity at the subtype level. This specificity of the primers was confirmed by sequencing the PCR products and by comparison to previously published sequences (Table 2.3) and/or by analysis of restriction enzyme digests of these products (Figure 2.3). The sequencing was done employing a number of different techniques. The first sequencing experiments were done by cloning the PCR products into a T-vector (Marchuk et al, 1991) containing a SP6 promoter and then using SP6 primers to sequence parts of the insert by the Sanger DNA sequencing method (Sanger et al, 1977). For one sequencing run (RARα), the Promega Silver Sequence kit (Promega Corporation) was used and subsequent sequencing of the PCR products was done with the Promega *fmol* DNA sequencing system (Promega Corporation). A detailed description of the sequencing methods will not be given here, because the methodologies are widely published and used, and the sequencing of the PCR products does not have a direct effect on the results of this study.

7. Polymerase chain reactions:

The semi-quantitative determination of RAR message by the use of PCR, a technique commonly known as RT-PCR (Chang et al, 1989), was based on the assumption that the reverse transcription (RT) reactions correctly mirror the relationship between a constitutively expressed control message and the expression of RARs in the

TABLE 2.2: Primer information for the three RAR subtypes and the four RNA loading controls used in the present study.

RNA	Primers (length in bases)	Product size (bp)	Base spanned	Reference
RARα	ACCGCCCACGCCACATGGA (19) TCTGTCCAAGGAGTCGCTGC (20)	169	1615-1783	Petkovich et al, 1987
RARβ (105)	CATGAACCCTTGACCCCAAG (20) CACGAGTGGTGACTGACTGA (20)	105	1580-1684	Benbrook et al, 1988
RARβ (208)	TAGCATCAGTGCTAAAGGTG (20) CACGAGTGGTGACTGACTGA (20)	208	1477-1684	Benbrook et al, 1988
RARγ	GCCTCTAGCGAGGATGAGGT (20) AGATGGTCAGTCTGCTGCCT (20)	123	1717-1839	Krust et al, 1989
β-actin	GCGTGACATTAAGGAGAAGCTG (22) CTCAGGAGGAGCAATGATCTTG (22)	375[a]	1200-1782	Nakajima-Iijima et al, 1985
riboso-mal PO	AGCAGGTGTTCGACAATGGC (20) CCTCCGACTCTTCCTTGGC (19)	345	649-993	Rich and Steitz, 1987
G3PDH	ACCACAGTCCATGCCATCAC (20) TCCACCACCCTGTTGCTGTA (20)	452	586-1037	Clontech[b]
Trans-ferrin recep-tor	CCACCATCTCGGTCATCAGGATT GCCT (27) TTCTCATGGAAGCTATGGGTATC ACAT (27)	1347	1227-2573	Clontech[b]

[a] Sequence reported in this reference is from genomic DNA and the primers span two introns. The genomic sequence spanned by the two primers is 582 base pairs (bp).

[b] Clontech Product Analysis Certificate for Glyceraldehyde 3-Phosphate Dehydrogenase Control Amplimer Set (catalog number 5405-1) and for Human Transferrin Receptor Control Amplimer Set (catalog number 5407-3) (Clontech Laboratories, Inc. Palo Alto, CA)

Table 2.3: Sequencing data from experiments confirming the identity of the PCR products.

PCR Product	Experimental (underlined) and published sequences
RARα	ACTGGGGtACCaTgTCCCTGGGGGGACtGGGGgcAGGGAGGAGGC $_{1728}$ACTGGGG ACC T TCCCTGGGGGAC GGGG AGGGAGGAGGC A C ACTC[a] AgCgACTC$_{1773}$
RARβ (105)	ATGAACCCTTGACCCCAAGTTCAAGTGGGAACACAGCAGAG $_{1581}$ATGAACCCTTGACCCCAAGTTCAAGTGGGAACACAGCAGAG CACAGTCCTAGCATCTCACCCAGCTCAGTGGAAAACAGTGGGG CACAGTCCTAGCATCTCACCCAGCTCAGTGGAAAACAGTGGGG$_{1664}$
RARβ (208)	TGCTGGAGAATTCTGAAG ACATGAACCCTTGACtCCCAAGTT $_{1560}$TGCTGGAGAATTCTGAAGgACATGAACCCTTGAC CCCAAGTT CAAGTGGGAACACAGCAGAGCACAGTC TAcgATCTCAC CAAGTGGGAACACAGCAGAGCACAGTCcTAgcATCTCAC$_{1640}$
RARγ	CCTCTAGCGAGGATGAGGTTCCTGGGGGCCAGGGCAAAGGGG $_{1718}$CCTCTAGCGAGGATGAGGTTCCTGGGGGCCAGGGCAAAGGGG GCCTGAAGTCCCCAGCCTGACCAGGGCCCCTGACCTCCCCGCT GCCTGAAGTCCCCAGCCTGACCAGGGCCCCTGACCTCCCCGCT$_{1802}$
β-actin	TCATGAAGTGTGACGTGGACATCCGCAAAGACCTGTACGCCAA $_{1513}$TCATGAAGTGTGACGTGGACATCCGCAAAGACCTGTACGCCAA CACAGTGCTGTCTGGCGGCACCACCATGTACCCTGGCATTGCC CACAGTGCTGTCTGGCGGCACCACCATGTACCCTGGCATTGCC$_{1598}$

[a]The RARα product was sequenced with the Silver sequence kit (Promega) in an attempt to use less radioactivity in these experiments. The RARα comparison exhibits a higher number of mismatches than the other sequencing experiments due to technical problems and thus difficulty to correctly read the

Table 2.3: (Continued).

sequencing gel. Lower case letters denote mismatches between the experimental and the published sequences. However, there was no doubt about the identity of the RARα product, since it was also verified by restriction enzyme digest analysis (see Figure 2.3).

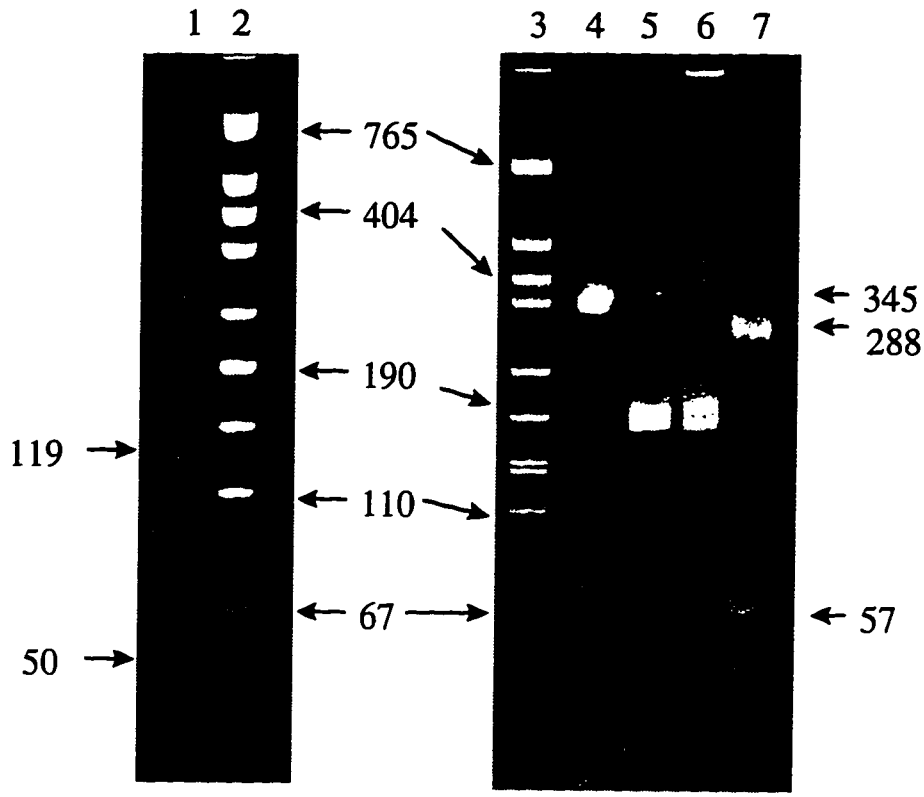

Figure 2.3: Restriction enzyme digests of RARα and ribosomal phosphoprotein (PO) PCR products confirm their identity. The PCR products were gel eluted and digested with appropriate restriction enzymes. The digests were fractionated on polyacrylamide gels (5% acrylamide for RARα and 7.5% for PO) and bands were visualized with ethidium bromide. Lanes 2 and 3 are size markers indicated as number of base pairs (bp) between the two gels. Expected sizes of the RARα fragments are indicated on the left and sizes of selected PO fragments are on the right. Lane 1 shows a RARα digest with Ava II - the expected fragments are 119 and 50 bp. Lane 4 is undigested PO PCR fragment - 345 bp. Lanes 5, 6 and 7 show digests of the PO fragment. In lane 5, a digest with Hinf I shows expected fragments of 170 and 167 bp. A small fragment of 8 bp is not visible on the gel. In lane 6, a digest with Ple I shows the expected fragments of 178 and 167 bp and in lane 7, a digest with Pvu II shows fragments of 288 and 57 bp.

different experimental conditions within each experiment. Ribosomal phosphoprotein (PO), glyceraldehyde-3-phosphate dehydrogenase (G3PDH), β-actin and transferrin receptor were all tested as constitutively expressed RNA controls. The β-actin message and the transferrin receptor were not found to be satisfactory controls due to variations in their expression with certain patterns of keratinocyte differentiation (see chapter VII). The most commonly used control in these experiments was ribosomal phosphoprotein (PO). In order to correct for differences in efficiency between the RT reactions, the first step of each PCR was to achieve uniform amplification of the control product PO between the various experimental conditions. The second step was to check that the amplification was within the exponential phase of the PCR. This was done by amplification of the PO control for a range of cycle numbers centered around the experimental cycle number in step one (e.g. 19 - 22 -25 cycles) and confirmation that the amount of product was increasing throughout this range. The third step was amplification of the RARs using the template loadings determined in step one. That the amplification was within the exponential range of the PCR for each product was checked as described above for PO. Each sample was also checked to insure the absence of genomic DNA by amplification of the total RNA sample without the RT. The amplified products were analyzed on 7.5% acrylamide gels and DNA visualized with ethidium bromide staining. Only after all the conditions (for specific PCR conditions see table 2.4) and controls were satisfactorily established, was the quantitative PCR performed. In these PCRs, the reactions were labeled with [α-^{32}P]dATP (Redivue^{TM}, Amersham, Arlington, IL). Radiolabeled dATP with specific activity of 3000Ci/mmol was used and each reaction was spiked with approximately 3μCi (1 pmol dATP). The amplified products were analyzed on 5% or 7.5% acrylamide gels which were dried and exposed to x-ray film (Amersham).

Table 2.4: PCR conditions.

Product	Denaturation		Annealing		Extension		Number
	Temp.	Time	Temp.	Time	Temp.	Time	of cycles
RARα	94°C	1 min	60°C	1 min	72°C	1 min	30
RARβ (105)	94°C	1 min	60°C	1 min	72°C	1 min	32
RARβ (208)	94°C	1 min	60°C	1 min	72°C	1 min	32
RARγ	94°C	1 min	60°C	1 min	72°C	1 min	28
β-actin	94°C	1 min	60°C	1 min	72°C	1 min	24
ribosomal PO	94°C	1 min	60°C	1 min	72°C	1 min	22
G3PDH	94°C	1 min	60°C	1 min	72°C	1 min	21
Transferrin receptor	94°C	1 min	60°C	1 min	72°C	1 min	32

8. RAR quantification:

The gels were analyzed quantitatively by phosphorimaging (Phosphorimager Facility, UW, Seattle, WA). Each set of products was always amplified with a negative control which included all reactants except the template. Expression of each product was then calculated as intensity of the specific band minus the negative control. Comparisons across the experimental conditions in each experiment were expressed as percent expression, where the most cornified experimental condition was set to 100%. Because of the large increases in RARβ expression under certain conditions, the comparisons were tabulated as log % expression.

9. RAR expression in oral tissues:

Cornified oral mucosa (from hard palate) and noncornified oral mucosa (from buccal vestibule) was removed from one patient and placed immediately on ice in Tissue Transport Medium (see above) and transferred from the clinic to the laboratory. Small pieces were cut off from each biopsy and processed for morphology and immunohistochemistry analyses (see above). The remaining pieces of the biopsies were placed in ice-cold 0.25% trypsin and incubated overnight at 4°C. The epithelium was then mechanically removed from the underlying connective tissues. Total RNA was extracted from the epithelial tissues only and processed for RAR quantification as described above for the cultured epithelia.

CHAPTER III

Modification of the organotypic raft cultures for growth of oral keratinocytes

1. Introduction:

Cell culture techniques provide many advantages for studies of cell differentiation over *in vivo* experiments. Two major such advantages are the availability of the cells for analysis and the ease of manipulating conditions of the cells' environment. However, a significant problem with cell cultures, especially with issues concerning cell differentiation, is the question of how true to life the growth conditions *in vitro* really are and whether results can be extrapolated to the *in vivo* situation? In the quest to approximate the growth of keratinocytes in culture as close as possible to their natural growth conditions, the organotypic raft (air-liquid interface) cultures have been developed. This methodology was first described by Bell et al (1979) as a model for studies of wound healing, but used extensively by Asselineau et al (1986, 1989 and 1995) for the study of epidermal keratinocyte differentiation. Prior to the start of this project there was no information on the growth and differentiation of human oral keratinocytes in this system. The first goal of this research was therefore to modify the organotypic raft culture model system for growth of human oral epithelial cells.

2. Preliminary experiments with foreskin keratinocytes:

Preliminary experiments consisted of an attempt to reconstruct the air-liquid interface culture model according to the technique of Asselineau and co-workers (for references see above). Consequently, a pilot project was initiated using foreskin keratinocytes. Following the protocol of Asselineau et al and using MEM10% (minimum essential medium with Earle's salts, without L-glutamine, with sodium bicarbonate and

with 10% fetal bovine serum), tertiary foreskin keratinocytes were seeded on collagen lattices containing GM10 fibroblasts (human embryonal epidermal fibroblast line). The keratinocytes were seeded on the contracted lattices with the help of stainless steel rings that kept the keratinocytes in immediate contact with a defined area of the collagen substrate and allowed them to attach. It was not clear whether the time allowed for the keratinocyte attachment would have any effects on the final product. A comparison between four hours and overnight attachment revealed no major differences in the morphology of the cultured epithelia (data not shown). However, it was practically easier to work with the short attachment time, because the steel rings tended to stick to the collagen lattices if left on overnight, and were consequently much harder to remove. Eventually, attachment times as short as two hours were used without any detrimental effect on the final differentiation of the epithelia.

In these conditions, the foreskin keratinocytes formed a stratified, but poorly differentiated epithelium after one week grown submerged in the medium (Figure 3.1). Adding one week at the air-liquid interface to these growth conditions, allowed these cells to form a cornified epithelium with a tendency to paracornification (few nuclei in stratum corneum were retained). In agreement with the morphology, these epithelia expressed markers of cornification (Figure 3.2). Keratin 1 (K1) was seen in single cells in the suprabasal cell layers and profilaggrin/filaggrin (proFG/FG) was expressed in the uppermost cornified layers, but in some places also revealed a granular cell layer. These results were comparable to the results published by Asselineau et al (1989).

3. Initial experiments with gingival keratinocytes:

The next step in the experimental protocol was to use gingival keratinocytes in this model system. Surprisingly, these keratinocytes behaved very differently. When grown in these conditions, they formed a stratified, but very poorly differentiated epithelium without expression of either proFG/FG or K1 (data not shown). It was

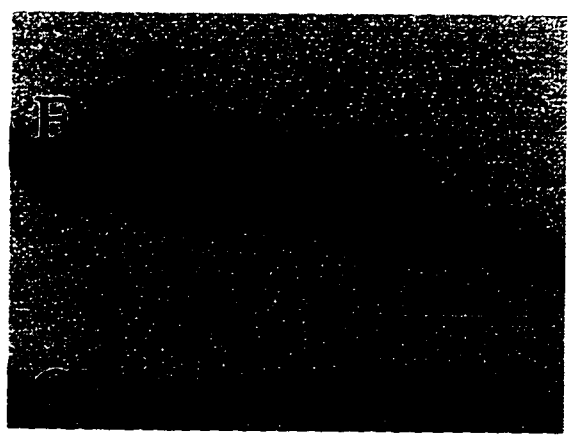

Figure 3.1: Foreskin keratinocytes form a stratified, but not well differentiated epithelium after one week of submerged culture. Foreskin keratinocytes were grown submerged for one week in MEM10% on collagen lattices containing GM10 fibroblasts. E marks the epithelium and CL marks the collagen lattice. The space between the two is a sectioning artifact. Bar is 100μm.

Figure 3.2: Foreskin keratinocytes form a cornified epithelium after one week at the air-liquid interface. Foreskin keratinocytes were grown on GM10 containing lattices for one week submerged in the medium (MEM10%) followed by one week elevated at the air-liquid interface. The organotypic cultures were fixed and sectioned and adjacent sections were stained with Hematoxylin and Eosin (H&E) for morphology and reacted with AKH-1 antibody for detection of profilaggrin/filaggrin and with 5C10 antibody for detection of keratin 1. Dotted lines on the left hand side of the two sections probed with antibodies mark basal borders of the epithelia. Bar is 100μm.

thought, that these keratinocytes may need a longer time for maturation at the air-liquid interface. However, extending the time by one week in the elevated position (i.e. two full weeks at the air-liquid interface) did not radically change the morphology or the expression of markers of cornification in these epithelia (Figure 3.3). It seemed that the gingival keratinocytes had different growth requirements than the foreskin cells.

At the same time, a group of scientists here in Seattle were doing work on human papilloma virus and keratinocytes, and they were using a similar, yet different, organotypic raft culture model for their studies. Their raft culture model was subsequently described by Hurlin et al (1991). In a trial to achieve better and more *in vivo* like differentiation, gingival keratinocytes were grown in the media utilized by this group[1]. This medium was DMEM10% with several "hormone" additives as described in Chapter II (p. 23), except that initially it also contained triiodothyronine (T3) (Sigma Chemical Co.) which was used at a final concentration of $2x10^{-11}$ mol/L. When gingival keratinocytes were grown in this medium and allowed to differentiate at the air-liquid interface for two weeks, a para/orthocornified epithelium was formed. In comparison to gingival tissues (Figure 3.4), this epithelium was more compact and lacked the prominent epithelial ridges seen *in vivo*. However, this epithelium did contain the four characteristic layers of normal gingival epithelium, i.e. the basal, spinous, granular and cornified cell layers. A partially dissociated zone of cells was seen above the well differentiated cells in most cultures. These cells were most likely formed early in the culture period prior to exposure to differentiating conditions at the air-liquid interface and remained associated with the differentiated culture due to lack of forces that normally cause desquamation *in vivo* (Lillie et al, 1988). Three representative markers of differentiation were localized by immunohistology to confirm the differentiation pattern indicated by the morphology of this epithelium (Figure 3.4). In the tissue, as well

[1] Acknowledgment and many thanks go to Dr. Pretender Kaur who taught me the secrets of the Dermal Equivalent system described by Hurlin et al (1991).

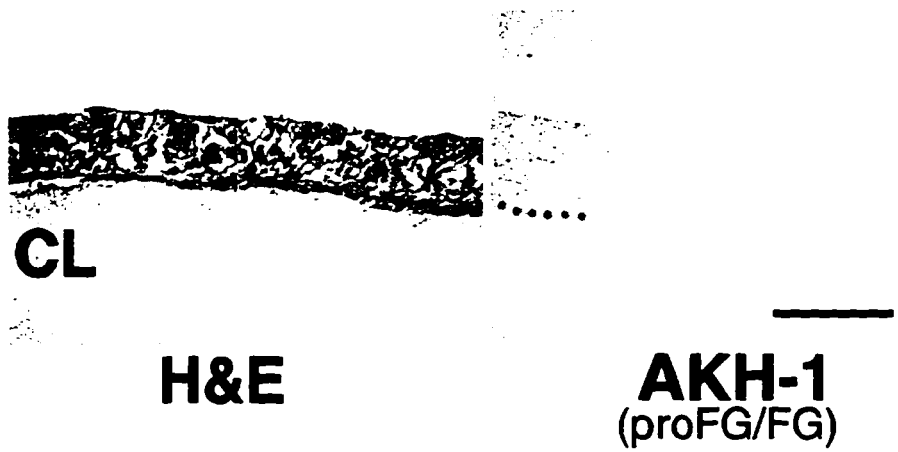

Figure 3.3: Gingival keratinocytes form a poorly differentiated epithelium after two weeks at the air-liquid interface when grown in MEM10%. Gingival keratinocytes were grown on GM10 containing lattices for one week submerged in MEM10% culture medium and then elevated to the air-liquid interface for 2 weeks. The cultures were fixed and sectioned and adjacent sections were stained with Hematoxylin and Eosin (H&E) or reacted with the AKH-1 antibody for detection of profilaggrin/filaggrin expression. E = epithelium and CL = collagen lattice. Dotted line marks the basal border of the epithelium and bar is 100μm.

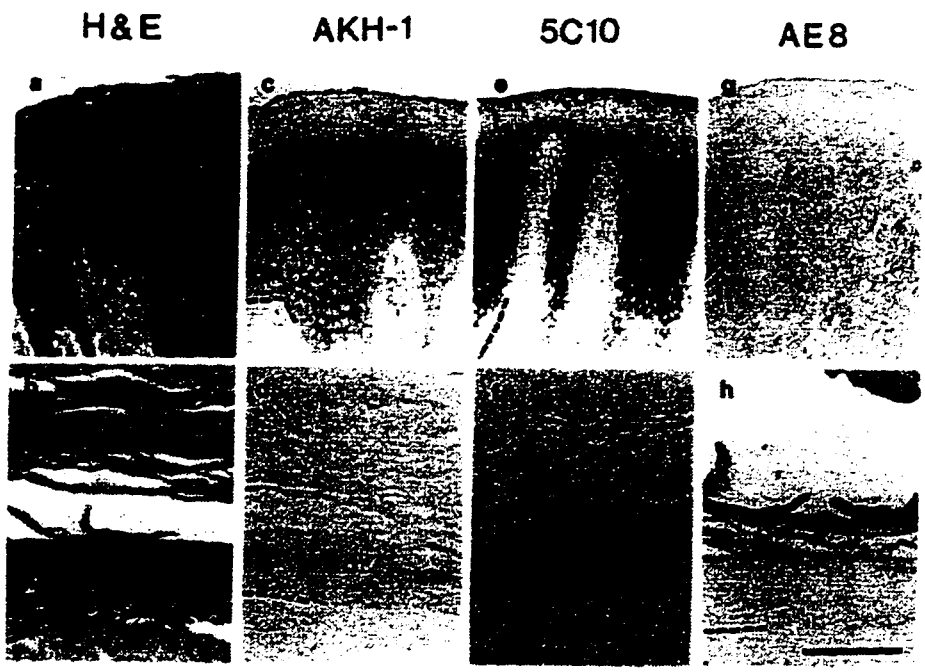

Figure 3.4: Gingival keratinocytes form a cornified epithelium in vivo as well as in vitro (in the organotypic raft culture). Gingival epithelium (a,c,e and g) is compared to epithelium formed by gingival keratinocytes grown in the organotypic raft culture (b,d,f and h). The cells were grown on GM10 lattices in DMEM10% including supplements as described in Material and Methods plus $2x10^{-11}$mol/L T3. The epithelium was formed during a period of three weeks (one week submerged and two weeks at the air-liquid interface). Tissue and cultures were fixed, sectioned and adjacent sections were stained with Hematoxylin and Eosin (H&E) or probed with antibodies AKH-1, 5C10 and AE8 to detect expression of profilaggrin/filaggrin, keratin 1 and keratin 13, respectively. Interrupted lines mark basal borders of the epithelia and bar is 100μm.

as in the cultured epithelium, the granular cell layer was delineated by reaction with antibody AKH-1, showing a band of proFG/FG expression immediately below the cornified cell layer. In agreement with the overall morphologic comparison between the two epithelia, the proFG/FG band in the cultured epithelium was thinner and expression seemed weaker. Both epithelia expressed K1 suprabasally as detected by antibody 5C10, but once again the band of K1 expression was thinner in the cultures. In contrast, K13, detected by antibody AE8, was expressed only in an occasional, suprabasal cell in the tissue and its expression was practically absent in the differentiating cell layers of the cultured epithelium. Some reactivity for AE8 was seen in the uppermost, desquamating cells.

During the course of these experiments, it was observed that differentiation of oral keratinocytes in the organotypic culture model was extremely sensitive to culture conditions prior to the growth on the lattices. Epithelial cells of higher passage number (Figure 3.5) or those that grew slowly in primary and secondary cultures (data not shown) differentiated poorly when transferred to the lattices. As illustrated in Figure 3.5a, second passage (tertiary) gingival keratinocytes formed a well differentiated paracornified epithelium displaying the four cell layers as discussed above. In contrast, third passage (quaternary) cells of the same origin and grown under the same conditions formed a noncornified-like epithelium lacking the granular and cornified cell layers (Figure 3.5b).

4. Conclusions from initial experiments; establishment of culture conditions for oral epithelial differentiation.

The preliminary experiments showed that organotypic raft cultures were a suitable model for growth of well differentiating gingival epithelia *in vitro*. However, it was evident that culture requirements were different for the oral cells than the previously published conditions for growth of foreskin keratinocytes. Furthermore, the growth

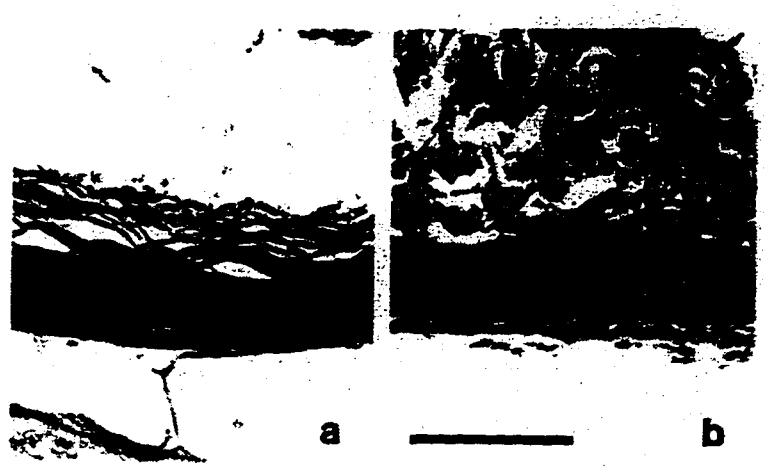

Figure 3.5: Gingival keratinocyte differentiation is dependent on passage number of the cultured cells. Tertiary (a) and quarternary (b) gingival keratinocytes were grown on GM10 fibroblast containing collagen lattices in medium with 10% normal (nondelipidized) serum. Bar is 100μm.

conditions of the oral cells prior to the seeding on the collagen lattices was important as well for their final differentiation. These observations suggested the necessity for using standardized conditions for growth and expansion of these cells. Therefore, unless otherwise stated, the subsequent experiments were done with second passage (tertiary) keratinocytes expanded for approximately two and a half weeks and then grown on the collagen rafts as described in Chapter II. In spite of these precautions a certain variability between consecutive experiments was noted and this was primarily attributed to the use of fresh keratinocyte cultures for each experiment. Consequently, results shown in this report were derived from comparisons within single experiments and any direct comparison between experiments was done with extreme caution.

CHAPTER IV

Retinoic acid effects on oral keratinocyte differentiation in organotypic raft cultures

1. Introduction:

The second major objective in this project was to investigate the effects of retinoic acid (RA) concentration on the differentiation patterns of oral keratinocytes in culture. Two hypotheses made up the basis for the experiments: First, it was hypothesized that differentiation of oral keratinocytes would be affected by RA in a similar fashion to the effects of RA on epidermal differentiation (Asselineau et al, 1989, see Chapter I). Second, based on the main hypothesis that oral regional variation in epithelial differentiation is due to a differential expression of the RARs (see Chapter I), it was predicted that oral keratinocytes from different regions of the mouth would exhibit specific reactivities to RA. Hence the specific aims of this part of the project were:

1. To investigate the effect of various RA concentrations on the differentiation patterns of oral keratinocytes in culture.
2. To compare the *in vitro* RA sensitivity of oral keratinocytes from divergent regions of the mouth.

In order to achieve these aims, the empirically derived culture conditions as described in the previous chapter were used in the subsequent experiments.

2. RA effects on oral keratinocyte differentiation *in vitro*:

Gingival keratinocytes (see Chapter II) were grown on GM10 containing collagen lattices in 0, 10^{-10}, 10^{-9} and 10^{-8}mol/L RA in medium containing delipidized serum (Figure 4.1) and in medium containing normal serum without any addition of RA

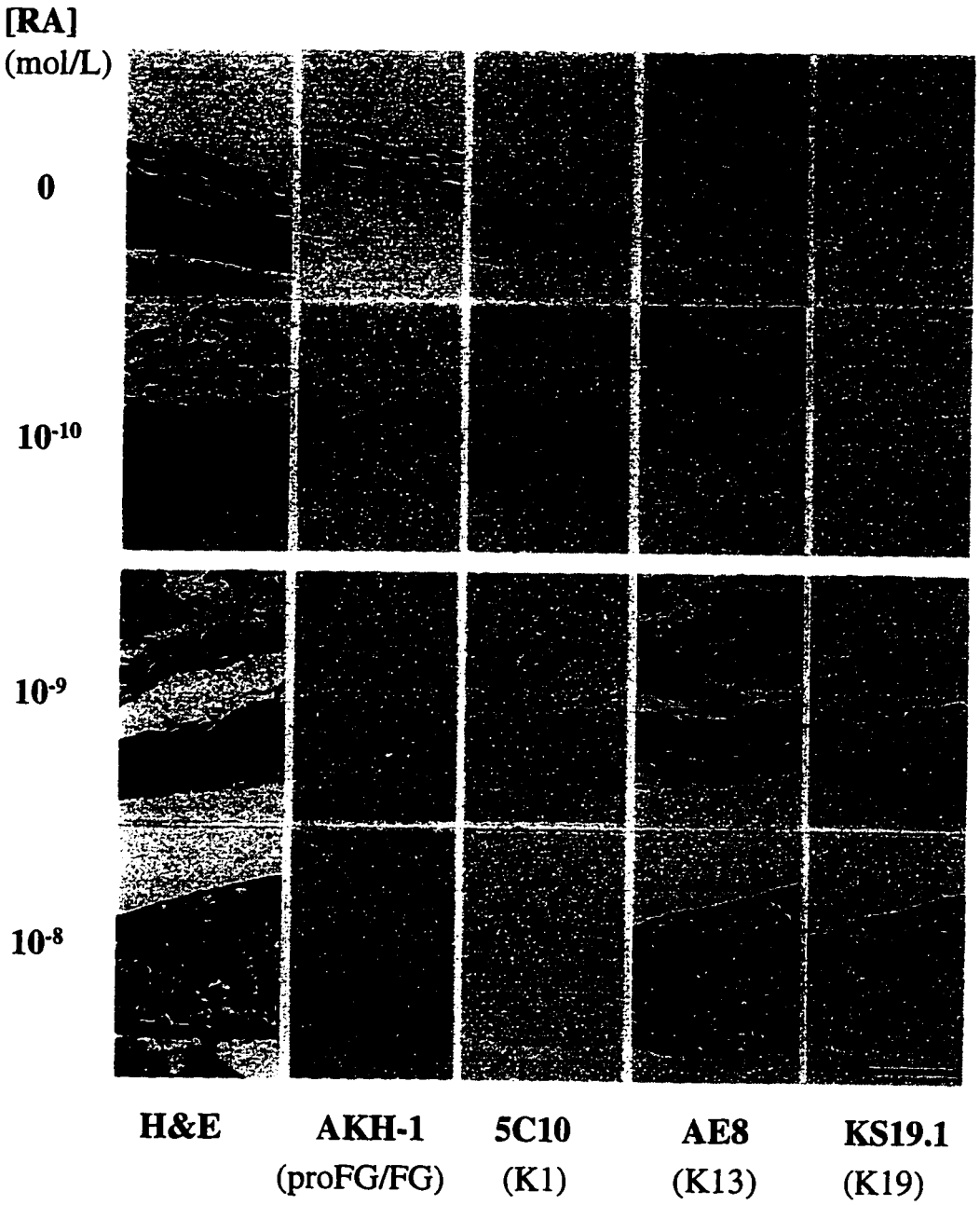

Figure 4.1: RA inhibits terminal differentiation of gingival keratinocytes in organotypic culture - immunohistochemistry. Gingival keratinocytes were grown on lattices containing GM10 fibroblasts in various RA concentrations as indicated. Epithelia were fixed, sectioned and stained with Hematoxylin and Eosin for morphology and with antibodies AKH-1, 5C10, AE8 and KS19.1 for detection of expression of markers of differentiation as indicated. Interrupted lines show basal epithelial borders. Bar is 100μm.

(data not shown). The latter condition was used as a control for the serum delipidization, ensuring that no detrimental quality changes of the serum occurred during this process. Analysis of the retinoid content in the control serum was estimated to be 148mg/L retinol, which is equivalent to approximately $5x10^{-7}$mol/L retinol in the 10% medium. In this particular experiment, keratinocytes grown in the control conditions differentiated similarly to the 10^{-8}mol/L RA conditions.

Figure 4.1 shows the effects of RA on gingival differentiation *in vitro*. In the absence of RA, a stratified squamous epithelium was formed. This epithelium expressed markers of cornified epithelia, profilaggrin/filaggrin (proFG/FG) and keratin 1 (K1), but the expression of the two markers of noncornified epithelia, K13 and K19, was virtually absent. Although, K13 was expressed in the uppermost cell layers of the cultured epithelium, these cells were presumably formed early in the differentiation process of this epithelium and have simply remained on top of the well differentiated part of the epithelium due to lack of desquamative forces (see chapter III). Of importance is that the differentiating layers of this epithelium expressed K13 weakly and only in single cells. This pattern corresponds well to the expression of this keratin in the cornified oral epithelium (see figure 3.4). When RA was added to the culture medium at a concentration of 10^{-10}mol/L, the gingival keratinocytes formed an epithelium that closely resembled the previous condition. This epithelium was also cornified and expressed the two markers of cornification, but did not express K19 and expressed K13 weakly. In media containing 10^{-9}mol/L RA, the oral cells also formed a stratified epithelium, but this epithelium lacked morphologic signs of cornification. In agreement with the morphology, this epithelium expressed no proFG/FG and K1 was expressed weakly and only in single cells. In contrast, there was an abundant expression of K13 in the suprabasal cell layers with the exception of one or two cell layers closest to the basal cells. In addition, K19 was also induced in the suprabasal cell layers of this epithelium. Although, the levels of expression of these four markers of differentiation corresponded well to the patterns of expression in oral noncornified epithelia, the site of K19

expression differed markedly, since K19 is most abundantly expressed in the basal cell layer *in vivo* (Sawaf et al, 1991, also see Figure 4.4). (Interestingly, in a histiotypic air-liquid interface culture system, Shabana et al (1991) cultured noncornified-like oral epithelia with the same site aberration in K19 expression, i.e. suprabasal versus basal). Finally, when RA was added to the media to a concentration of 10^{-8}mol/L, the gingival keratinocytes formed a multilayered, but not well stratified epithelium. The disorganization of epithelial morphology is viewed as a sign of RA toxicity on these epithelial cells. Interestingly, in this epithelium with a total lack of cornification and in spite of its poor morphology, the four markers of epithelial differentiation were correctly expressed in a quantitative sense. The two markers of cornified epithelia, proFG/FG and K1 were absent, while the two markers of noncornified epithelia, K13 and K19, were abundantly expressed. Both these markers were expressed in all suprabasal cell layers.

The immunohistochemistry results were substantiated by immunoblotting experiments for three of the four markers used, namely proFG/FG, K1 and K19 (Figure 4.2). (Detection of the fourth marker, K13, was at the time of these experiments not successful, but it was later shown that results from immunoblots with K13 correspond well with the immunohistochemistry data (see Figure 6.8)). On equally loaded blots, as determined by Coomassie brilliant blue staining of proteins on a parallel gel, K19 expression was increased as the RA concentration and thus the tendency to noncornification was increased. Conversely, K1 and proFG/FG expression was decreased in the same conditions. When proFG was expressed in these culture conditions (0 mol/L RA and 10^{-10}mol/L RA), its processing to FG was weak. The blots also show that expression or lack of expression of the three markers was similar in the control cultures (media containing normal serum) and in the 10^{-8}mol/L RA conditions.

The RA inhibition of oral epithelial cornification was subsequently reproduced in more than ten separate experiments (not all of these experiments had the primary aim to reproduce the RA effect, but were done for other reasons). In each of these experiments, primary keratinocytes from different regions of the oral cavity were used

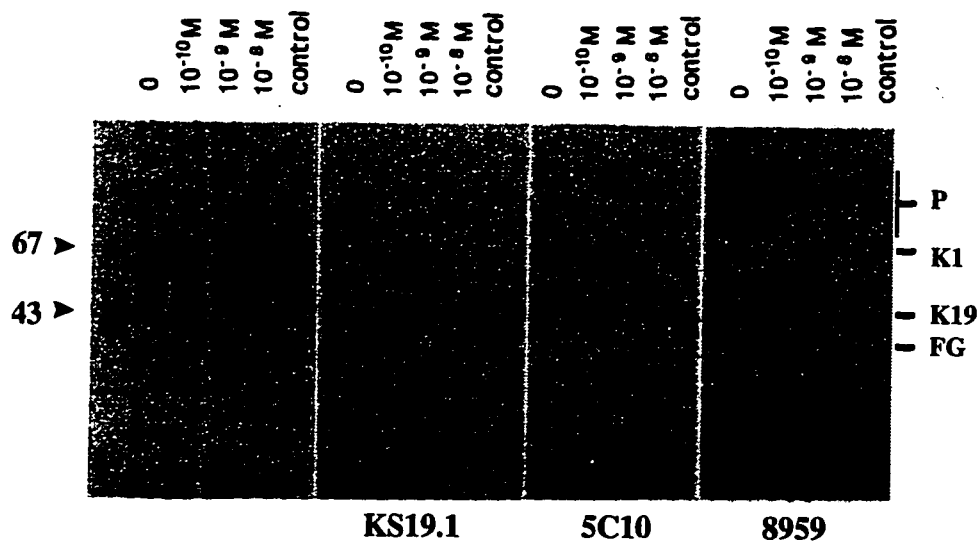

Figure 4.2: RA inhibits terminal differentiation of gingival keratinocytes in organotypic culture - immunoblots: Epithelia grown in parallel to those shown in Figure 4.1 were used for protein extraction and immunoblotting as described in Chapter II. RA concentrations used in this experiment are shown above each lane. The control condition contained normal serum in the medium and approximated the 10^{-8}mol/L RA condition. The first panel is a Coomassie Blue stained gel, the other panels are duplicate gels that were blotted and reacted to antibodies as shown below each panel. The first lane in the first panel shows size markers, two of which are marked on the left in kiloDalton. The proteins detected by the antibodies are shown on the right; P = profilaggrin, K1 = keratin 1, K19 = keratin 19 and FG = filaggrin.

and this experimental approach contributed to two observations: First, that the absolute RA sensitivity differed between the different epithelial cell lines and second, that this characteristic did not seem to be dependent on the origin of these cells, i.e. whether they originated from cornified or noncornified oral epithelium.

3. Absolute RA sensitivity of oral keratinocytes:

Fresh keratinocytes from different donors were used for each subsequent culture experiment. The decision to use primary keratinocytes was based on the fact that the ability of the cells to terminally differentiate decreased with increasing passage number (see Chapter III). It was feared that the process of expanding a primary keratinocyte culture, freezing it in aliquots, thawing it and reexpanding the cells for the final experiments would not provide cells with low enough passage number, thus sacrificing terminal differentiation of the cultured epithelium.

In nine consecutive experiments, using primary keratinocytes from both noncornified and cornified oral mucosa, the cultured epithelia exhibited various degrees of cornification independent of their region of origin (Table 4.1.). Figure 4.3 shows the variation in degree of cornification for the nine keratinocyte strains when grown in media containing 10% normal (nondelipidized) serum. Morphology of the control condition in each experiment is shown, because this was the one condition used in all of the early experiments in this project. As discussed above, the control condition approximated the 10^{-8}mol/L RA cultures in these experiments. At this RA concentration, the cultured epithelia ranged from paracornified epithelia to poorly organized epithelia without any signs of cornification and there was no particular pattern to this variation in regard to the origin of the cells. These results suggested that degree of cornification and hence, degree of RA sensitivity was dependent on other factors than the original differentiation pattern of the oral keratinocyte. The wide variation in RA sensitivity of each primary

Table 4.1: Correlation between the original (*in situ*) differentiation of the epithelial tissues and the corresponding pattern of the epithelial differentiation in culture (*in vitro*).

	***In situ* differentiation**		
***In vitro* differentiation[a]**	**Cornified**	**Noncornified**	**Not determined**
Cornified	1 (exp. 26)	1 (exp. 33)	-
Noncornified	-	1 (exp 29)	-
Noncornified/poorly organized	1 (exp. 91-17B)	3 (exps. 27, 31 and 91-17A)	2 (exps. 30 and 32)

[a] The *in vitro* differentiation data were taken from the "control" conditions in each experiment and differentiation was judged on the basis of epithelial morphology (see Figure 4.3) and immunohistochemistry experiments (data not shown).

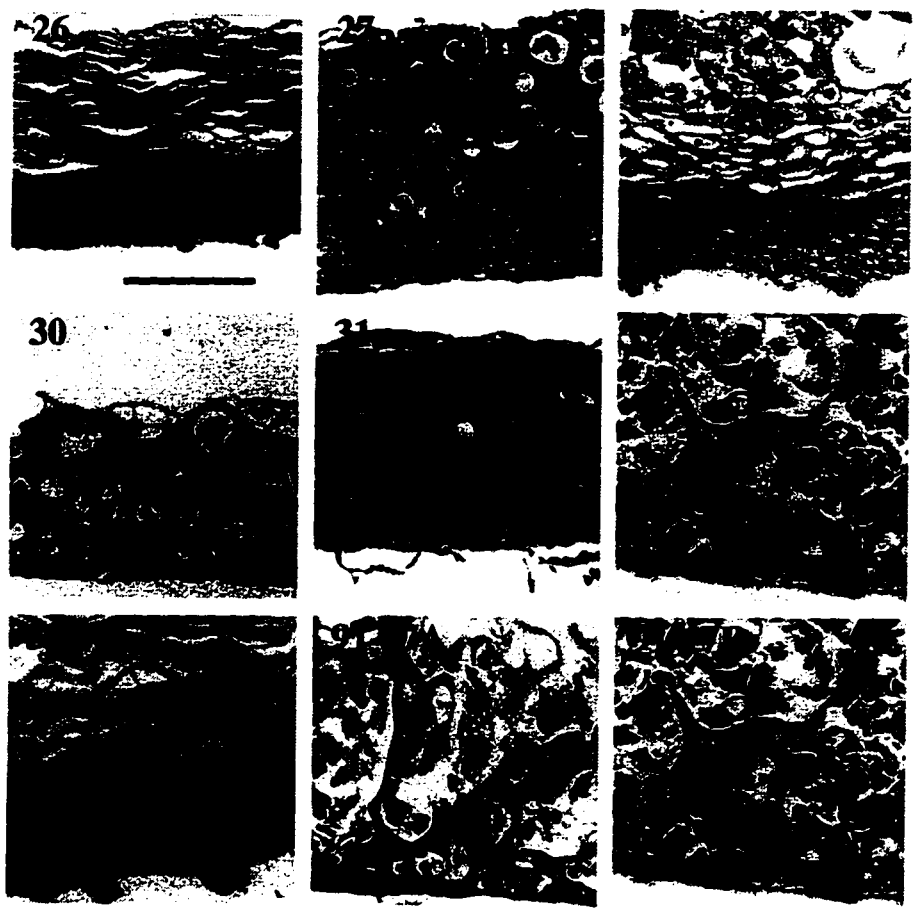

Figure 4.3: Degree of epithelial cornification *in vitro* does not correlate with the original *in situ* differentiation of the epithelial cells. Hematoxylin and Eosin stained sections from nine consecutive experiments (experiment 26 - experiment 91-17B) are shown. All cultures were grown as described in Chapter II in media containing normal serum and on lattices with GM-10 fibroblasts. Keratinocytes from cornified regions were used in experiments 26 and 91-17B. Keratinocytes from noncornified regions were used in experiments 27, 29, 31, 33 and 91-17A. Origin of the keratinocytes used in experiments 30 and 32 was not reliably determined. Bar in upper, left section is 100μm.

keratinocyte culture is probably due to several factors, some of which may be the age and sex of the tissue donor, the amount of inflammation in the original tissue, the primary culture conditions of the cells, etc. As will be shown in Chapter V, the underlying fibroblasts have distinct influence on the apparent RA sensitivity of the keratinocytes. However, these nine consecutive experiments were all cocultured with the same fibroblast type, GM10, and therefore this explanation for the variation in RA sensitivity seems unlikely.

4. Effect of the *in situ* pattern of epithelial differentiation on RA sensitivity of oral keratinocytes in the organotypic raft culture:

Because of the large variation in the apparent RA sensitivity of various oral epithelia, it was of interest to compare directly how oral keratinocytes from a cornified and a noncornified region would react to RA. However, being at the mercy of the oral surgeon for the kind of tissues made available for the organotypic culture experiments, made planning of such experiments very difficult. Because of the large inter-experimental variation, possibly dependent on several factors as discussed above, the ideal experiment to answer this question would utilize tissues of two separate and divergent differentiation patterns from one patient and be performed coordinately in time. Opportunity for such an experiment arose when a surgery provided a large piece of oral mucosa from one patient. This tissue was clearly demarcated macroscopically by the mucogingival border and was sectioned along this border, before being converted into two separate oral epithelial cultures as described in Chapter II. The two macroscopically different epithelial differentiation patterns were verified microscopically by morphology and immunohistochemistry. Figure 4.4 shows that tissue 92-9A was covered by an orthocornified epithelium expressing proFG/FG in the granular and cornified cell layers and K1 suprabasally, while K13 expression was limited to certain

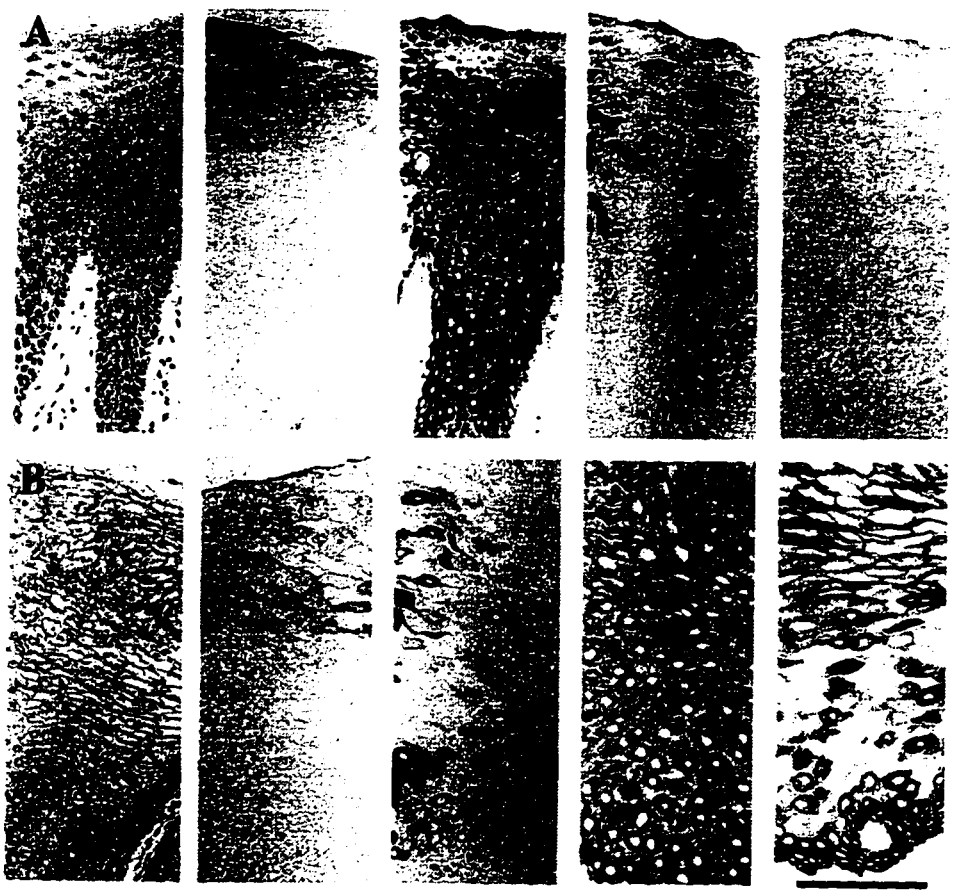

Figure 4.4: Tissue 92-9A (A) is covered by a cornified epithelium, while tissue 92-9B (B) is covered by a noncornified epithelium. Histological sections from the two tissues were stained with Hematoxylin and Eosin for morphology and with antibodies AKH-1, 5C10, AE8 and KS19.1 for expression of proFG/FG, K1, K13 and K19, respectively. Bar is 100μm.

regions of this epithelium and K19 was not expressed at all. In contrast, tissue 92-9B was covered by a noncornified epithelium with virtually no or limited expression of proFG/FG and K1, while expressing K13 in all suprabasal cells and K19 especially in the basal cell layer.

Keratinocytes from these two tissues were grown in parallel on rafts in media containing no RA and in media containing 10^{-8}mol/L RA. The morphology and expression of markers of differentiation in the cultured epithelia are shown in Figure 4.5. In RA-free conditions, both cell types formed a parakeratinized, stratified epithelium with similar expression of proFG/FG, K1 and K13. In both epithelia, probing for proFG/FG revealed a distinct granular cell layer and reactivity was also seen in the uppermost, desquamating cells. K1 was expressed as a band in the upper spinous and the granular cell layers and K13 expression was practically absent. In 10^{-8}mol/L RA, both keratinocyte types formed an epithelium that was less well keratinized, but again morphology and expression of proFG/FG, K1 and K13 were comparable. In this condition, proFG/FG was expressed only in the uppermost, squamous cells and a distinct granular cell layer was not seen. K1 was expressed only in single cells at the level of the upper spinous cell layers. K13 expression was limited to the uppermost cell layers, without significant involvement of the actively differentiating parts of the epithelia. In contrast, expression of K19 differed consistently between these two epithelia in both RA concentrations and in other growth conditions (data not shown). In absence of RA, K19 was virtually absent from the epithelium originating from the cornified mucosa, while there was a distinct, albeit patchy expression of K19 in the epithelium originating from the noncornified area. In fact, K19 was more abundantly expressed in the latter epithelium, than in epithelium originating from the cornified region and grown in the presence of 10^{-8}mol/L RA. Taken together, K19 expression was consistently higher in the cultured epithelia originating from noncornified oral mucosa, mimicking the pattern of expression seen in the original tissues [Figure 4.4 and Sawaf et al, 1991].

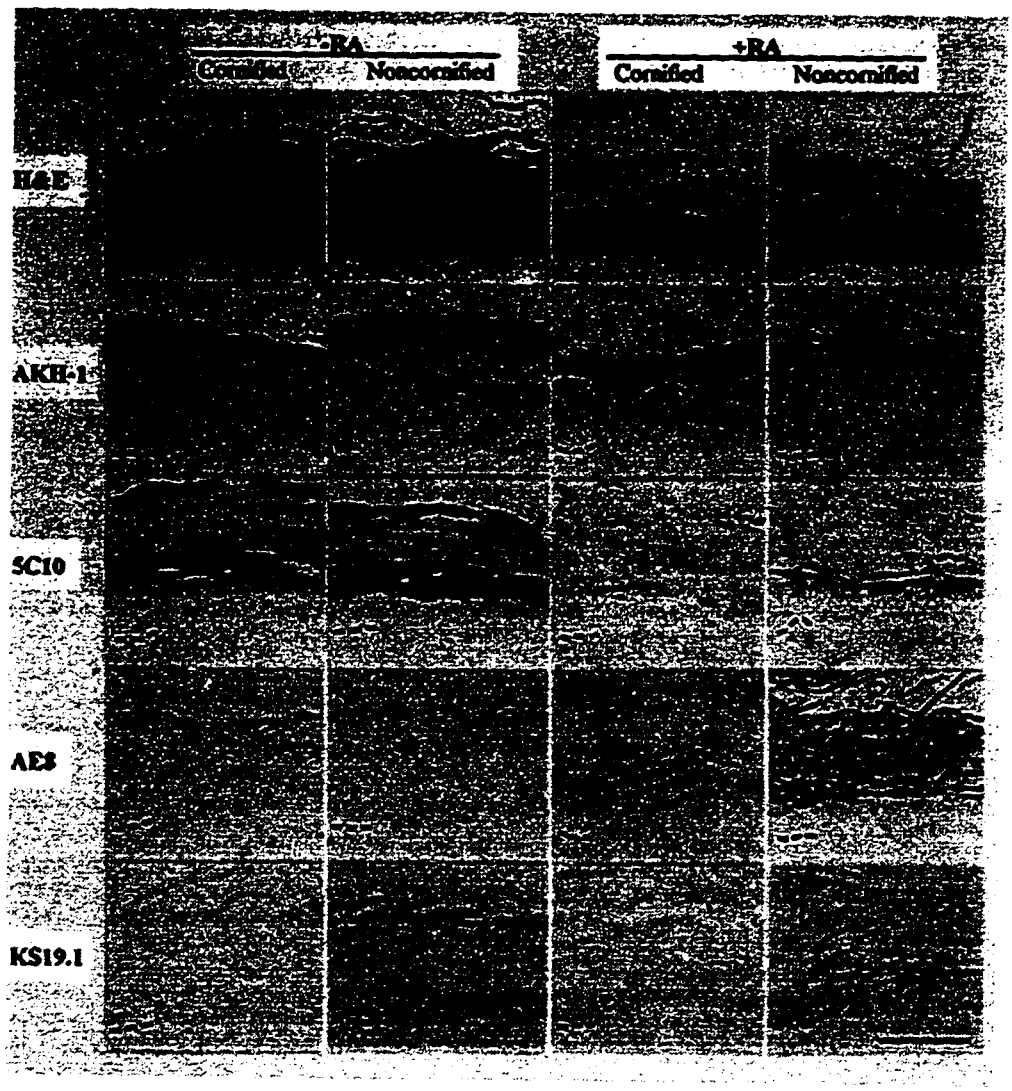

Figure 4.5: Keratinocytes from cornified and noncornified oral epithelia differentiate similarly in response to RA in organotypic cultures. Keratinocytes from the tissues shown in Figure 4.4 were grown in retinoid free conditions (-RA) and in media containing 10^{-8}mol/L RA (+RA). Cultures were fixed, sectioned and stained as described in Chapter II. H&E shows morphology of the epithelia, and staining with AKH-1, 5C10, AE8 and KS19.1 shows expression of proFG/FG, K1, K13 and K19, respectively. Interrupted lines show basal borders of the epithelia. Bar is 100μm.

5. Summary and conclusions:

Addition of RA to the culture medium inhibited cornification of oral keratinocytes and promoted a noncornified-like pattern of differentiation in the cultured epithelia. Interestingly, oral keratinocytes taken from the same patient, but originating from cornified and from noncornified epithelia showed a similar response to RA, except in the expression of K19. In contrast, oral keratinocytes taken from different donors displayed a wide range in the apparent RA sensitivity. These results indicate that oral keratinocytes from adjacent cornified and noncornified regions are, as a whole, not fundamentally different cell types, but are receptive to environmental directives for their terminal differentiation. However, certain aspects of the two cell types, such as K19 expression, may be genetically predetermined.

CHAPTER V

Retinoic acid and triidothyronine interactions

1. Introduction:

The material presented in this chapter is somewhat peripheral to the main thrust of this project. However, since other investigators use keratinocyte culture media containing triiodothyronine (T3) and there is a theoretical basis (see below) for possible interactions between retinoic acid (RA) and T3 in the cultured epithelia, it was of concern that T3 may have significant effects on the RA-sensitive differentiation of oral keratinocytes *in vitro*. Experiments attempting to define a possible RA/T3 interaction in oral keratinocytes grown in the organotypic cultures are described in this chapter.

2. Background:

In the past decade much progress has been made in our understanding of the mechanisms by which transmembrane diffusable hormones affect cellular functions. Much of this knowledge was facilitated by the discovery of a superfamily of proteins (steroid/thyroid hormone receptors), which are able to bind hormones and DNA and through this interaction function as transcriptional regulators (Evans, 1988; Mangelsdorf et al, 1995). In accordance with this function, these proteins contain a well conserved central domain responsible for DNA binding and a C-terminal domain which is important in recognition and binding of specific ligands (compare with review of RARs in Chapter I). The central domain exhibits an array of cysteine residues forming two so-called zinc fingers and these structures mediate the recognition and binding of specific DNA sequences, known as response elements (REs). Many of the proteins in this superfamily display a high amino acid sequence identity in this domain. For example, there is a 62%

amino acid sequence similarity between the human beta thyroid hormone receptor (hTRβ) and the human retinoic acid receptor alpha (hRARα) in this region (Glass et al, 1989). Although, specificity for receptor recognition of REs exists and is determined by both sequence of the RE and other structural characteristics, such as spacing between half-sites of the RE sequence (Mangelsdorf and Evans, 1995), there is also a considerable overlap in RE recognition between several of the receptor proteins. Such overlap has been described for RARs and TRs (Umesono et al, 1988), for RARα and vitamin D receptor (Schule et al, 1990) and for TRs and the estrogen receptor (Sharif and Privalsky, 1991).

A second level of interaction exists at the protein level of these receptors (Mangelsdorf and Evans, 1995). Except for the steroid receptors, all other known ligand dependent receptors in this superfamily heterodimerize with retinoid X receptors (RXRs). This mechanism provides an indirect pathway of interaction between the TRs and the RARs. Such interactions have been observed in TRβ mutated animals which exhibit several defects other than those of thyroid hormone deprivation (Collingwood et al, 1994). It is hypothesized that these TR mutants interfere with events not normally mediated by TRs by RXR titration. A more direct interaction may occur by direct heterodimer formation between TRs and RARs. Using crosslinking methodology, Glass et al (1989) showed that such heterodimers exhibit novel transcriptional properties. The overlapping RE recognition and the direct or indirect protein-protein interactions provide a theoretical basis for a complex network of transcriptional control of RA and T3 responsive genes.

Although the significance of RAR/TR interactions *in vivo* is not well understood, a wide range of such interactions has been observed in a variety of more or less physiologically relevant models. Morita et al (1990) have demonstrated that both RA and T3 are able to stimulate growth hormone secretion in cultured rat pituitary cells. The stimulatory effects of these two hormones on growth hormone mRNA expression were additive in this system. Rohrer et al (1991) showed that both RA and T3 can

induce expression of certain genes, such as that of α-myosin heavy chain, in cultured rat cardiac myocytes. However, in this instance, no potentiation of the effect of the two hormones was evident and it was shown that the T3 and RA induction is mediated through distinct response elements and/or mechanisms. Ballerini et al (1991) showed that although thyroid hormones are not able to induce leukemic cell differentiation, in combination with RA, T3 is able to potentate RA-induced granulocytic differentiation by one order of magnitude. In contrast to these stimulatory effects on gene transcription, Hudson et al (1990) reported that the EGF receptor gene is inhibited by ligand-activated TRs and RARs in several cell lines. However, when both receptors were expressed in the presence of only one ligand, this inhibition was reversed. These examples of RA/T3 interactions exhibit a number of different effects on gene transcription, strengthening the impression that RA and T3 may be involved in a complex regulatory crosstalk and also illustrating that the specific RA and T3 effects are highly context dependent.

It has long been known that RA is a potent effector of epithelial differentiation (see Chapter I). Much less is known about the influence of T3 on epithelia. However, one of the clinical signs seen in hypothyroidism is scaly skin, suggesting that epidermis may be a specific target for T3 activity. Isseroff et al (1989), showed that epidermal cells grown in T3 depleted media exhibit increased levels of transglutaminase and increased formation of cornified envelopes. Both these phenomena indicate that T3 depletion stimulates the cornified type of epithelial differentiation, corresponding well to the clinical connection between hypothyroidism and increased skin scaliness. This contention is supported by work of Tomic et al (1990), who demonstrated that the genes of keratins 5, 6, 10 and 14 are all downregulated both by RA and T3 in three epithelial cell types - HeLa, rabbit corneal and rabbit esophageal epithelial cells. In contrast, K14 expression was augmented by the aporeceptor TRα (in absence of T3). However, the question of how RA affects keratin expression in combination with T3 was not addressed in this study.

As described in chapter III, the protocol for the raft cultures published by Hurlin et al (1991) called for addition to the medium of a growth hormone cocktail containing T3. In light of the above discussion, it was of concern that T3 in the growth media may strongly influence the outcomes of the RA experiments. Therefore, a series of experiments were performed in order to investigate the possible RA/T3 interactions in the organotypic raft cultures.

3. The experiments and results:

The initial observation that T3 may affect terminal differentiation and expression of differentiation markers of oral keratinocytes in the organotypic culture was derived from two separate experiments; one was done with media containing T3 at a final concentration of $2x10^{-11}$mol/L and one was done with media without any T3. Figure 5.1 shows the morphology and immunohistochemistry from these two experiments. In absence of RA, oral keratinocytes formed a paracornified epithelium in both conditions. However, the epithelium grown with T3 contained fewer nuclei in the cornified cell layers, thus approaching more closely the orthocornified state of differentiation than its counterpart grown without T3. There were no remarkable differences in expression of the two markers of cornification, proFG/FG and K1, in these two epithelia, although the proFG/FG layer was thinner in presence of T3, suggesting a more site specific control of expression of this protein. The one marker of noncornified oral epithelia examined in these experiments, K13, was slightly more abundant in the absence of T3. When RA was added to a final concentration of 10^{-8}mol/L, the differences between the T3 and no T3 conditions were accentuated. In this instance, T3 markedly inhibited the effects of RA as the epithelium grown with T3 showed a much higher degree of cornification than the epithelium grown without T3. In the absence of T3, the epithelium displayed a disorganized morphology with erratically scattered expression of proFG/FG and K1 and with abundant expression of K13 in all cell layers but the basal layer. In contrast, when

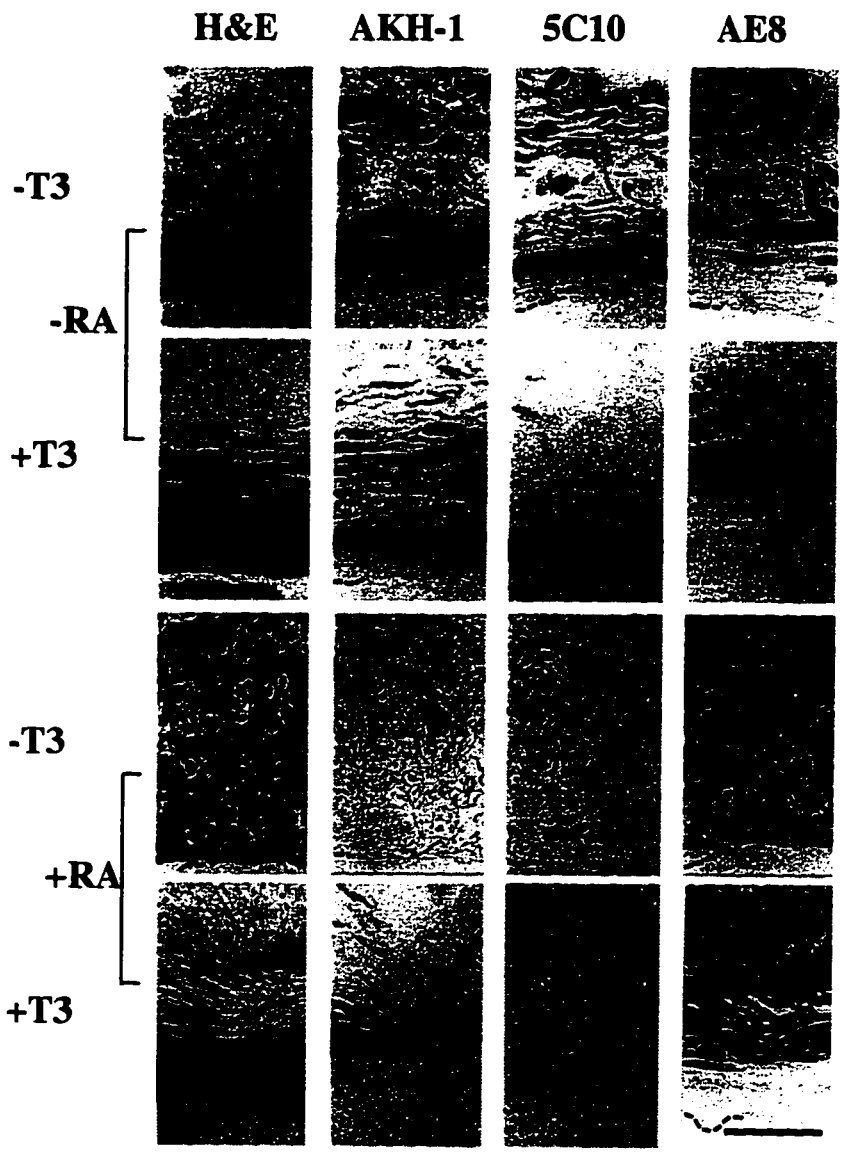

FIGURE 5.1: T3 decreases the apparent RA-sensitivity of gingival keratinocytes in both low and high RA concentrations in organotypic cultures - immunohistochemistry. Gingival keratinocytes were grown on GM-10 containing lattices in media without RA (-RA) and with 10^{-8}mol/L RA (+RA) and with or without addition of T3 ($2x10^{-11}$mol/L) as indicated. Cultured epithelia were fixed, sectioned and analyzed for morphology by H&E staining and for expression of differentiation markers by immunohistochemistry. AKH-1 detects proFG/FG expression, 5C10 detects K1 and AE8 detects K13. Interrupted lines mark basal epithelial borders and bar is 100μm.

T3 was present, the keratinocytes formed a paracornified epithelium with orderly expression of proFG/FG and K1 in the upper spinous cell layers and with practically no expression of K13 in the differentiating (spinous) layers. The notion that T3 diminishes the inhibitory effects of RA on oral epithelial cornification was further substantiated by immunoblotting (Figure 5.2). In this analysis, K19 was examined as the marker of noncornified differentiation. Its expression was decreased in presence of T3 in both low and high RA concentrations (0 mol/L and 10^{-8}mol/L). The quantitative expression of K1 was difficult to compare between the two T3 conditions in the low RA concentration, because there was a distinct qualitative difference in the expression of this marker of cornification. A distinct 67kDa band, corresponding well to the molecular weight of K1, was detected in extracts from the T3 condition. In contrast, extracts from T3-free conditions displayed several immunoreactive bands of lighter molecular weight. These bands may either be degradation products from K1 or they may indicate expression of a distinct, but immunologically cross reactive protein with an apparent molecular weight of 65kDa, which subsequently underwent proteolytic degradation. In the higher RA concentration, regardless of T3, the 67kDa band was not detected and the 65kDa band was very faint. There was also a distinct qualitative difference of proFG/FG expression in low RA concentrations in the two T3 conditions. Without T3, proFG expression was abundant, but there was no or little processing of this high molecular weight precursor to the 38kDa FG protein. In contrast, in cultures grown with T3, proFG was readily processed to FG. A clear quantitative difference was seen in the high RA concentrations, where keratinocytes cultured in the presence of T3 expressed more proFG than keratinocytes grown without T3. Taken together, these results clearly indicated that T3 opposes the apparent RA inhibitory effects on cornification of oral keratinocytes.

No or little difference in expression of markers of differentiation was seen in cultures grown with media containing normal serum (not delipidized) in conditions with or without added T3 (Figure 5.2, "control" lanes). The lack of T3 effect in these

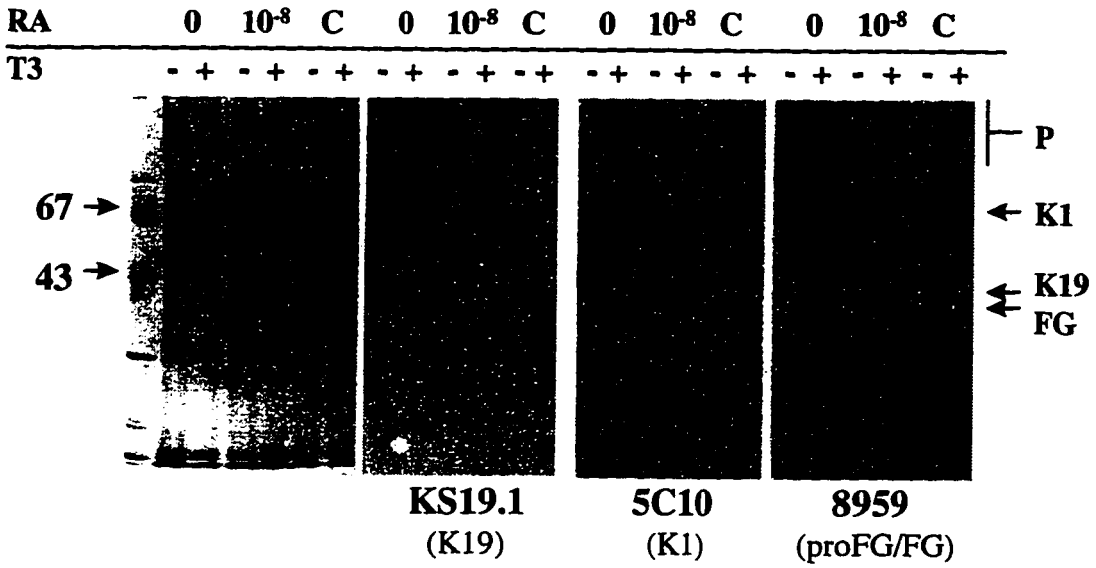

Figure 5.2: T3 decreases the apparent RA-sensitivity of gingival keratinocytes at both high and low RA concentrations in organotypic cultures - immunoblots. Epithelia grown in parallel to those shown in Figure 5.1 were used for protein extraction and immunoblotting as described in Chapter II. RA concentrations used in this experiment are shown above each pair of lanes and are expressed in mol/L. The C lanes show proteins from epithelia grown in media with normal (not delipidized) serum. Absence or presence of T3 at $2x10^{-11}$mol/L is indicated above each lane as - or +. The first panel is a Coomassie Blue stained gel, the other panels are duplicate gels that were blotted and reacted to antibodies as shown below each blot. The first lane in the first panel shows size markers, two of which are marked on the left in kiloDalton. The proteins detected by the antibodies are shown on the right; P = profilaggrin, K1 = keratin 1, K19 = keratin 19 and FG = filaggrin.

conditions was most likely due to the relatively high, endogenous T3 content of the normal serum. An analysis of the T3 content, revealed that the normal serum contained $1.6x10^{-9}$mol/L T3 (Table 5.1). At 1:10 dilution in the media, the final concentration of T3 was $1.6x10^{-10}$mol/L T3. Thus, add-back of T3 ($2x10^{-11}$mol/L) to the control medium raised the T3 concentration to a relatively negligible degree (by 3.3%). This is in contrast to the delipidized serum containing medium, in which the add-back of T3 increased its concentration by at least 33% (but most likely more; the exact increase of T3 cannot be determined since the original amount of T3 in the delipidized serum was below the detection level of the analysis) of the original T3 content.

When the organotypic raft culture model was used as described in Chapter II, the apparent RA sensitivity varied widely from experiment to experiment. This was most probably due to the use of primary keratinocytes for each experiment (for further discussion see Chapter IV). It was, therefore, of importance to follow up these initial data taken from two separate experiments and repeat the RA/T3 interactions within single experiments. Results from one such experiment are shown in Figure 5.3. Addition of T3 markedly reversed the RA inhibitory effects on oral keratinocyte cornification at a RA concentration of 10^{-9}mol/L. Without T3, a stratified, but not well cornified, epithelium was formed in culture. This epithelium expressed very little proFG/FG and K1, but showed some expression of K13 and K19. When T3 was added, the epithelium formed was clearly paracornified, with a distinct expression of proFG/FG in the granular layer and abundant expression of K1 suprabasally. The two markers of noncornified epithelia, K13 and K19, were practically absent from the differentiating layers of this epithelium. The increase in expression of the two markers of cornification in presence of T3 was also shown by immunoblots (Figure 5.4). In this experiment, there was again an increase of proFG processing to FG in the T3 conditions, but there was also an overall increase in proFG expression. This result clearly substantiated the preliminary data presented above. Interestingly, when the cells were grown in 10^{-8}mol/L RA in the same experiment, no apparent effect of T3 was visible (data not shown).

Table 5.1: Triidothyronine (T3) content in serum (analysis performed by the Clinical Nutrition Research Unit at the University of Washington) and in culture media (calculated).

Type of Fetal Bovine Serum	T3 in Fetal Bovine Serum (mol/L)	T3 in 10% DMEM (mol/L)	T3 after add-back (mol/L)
Normal	$1.6x10^{-9}$	$1.6x10^{-10}$	$1.8x10^{-10}$
Delipidized	$<0.6x10^{-9}$ [a]	$<0.6x10^{-10}$	$<0.8x10^{-10}$

[a] $0.6x10^{-9}$mol/L was the limit of T3 detection in this analysis.

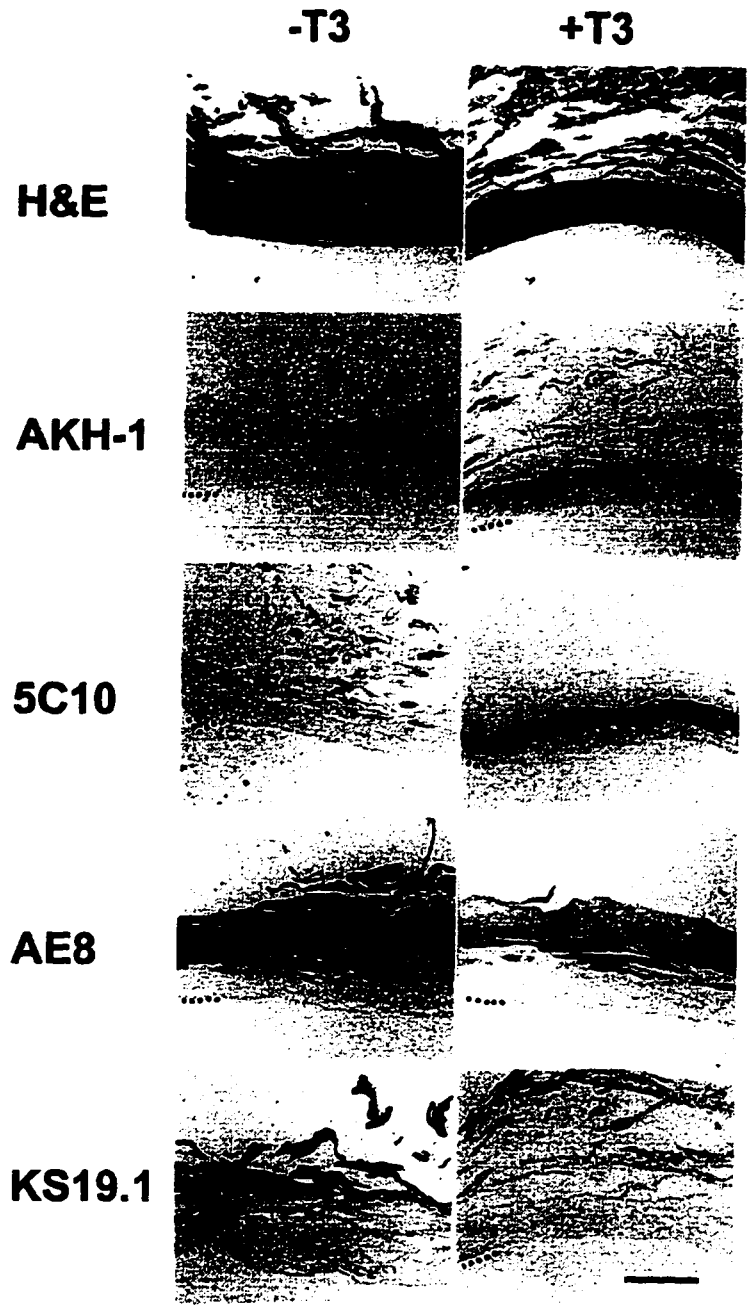

Figure 5.3: Intraexperimental confirmation of the T3 mediated decrease in the apparent RA sensitivity of gingival keratinocytes - immunohistochemistry. Gingival keratinocytes were grown in the organotypic culture in media containing 10^{-9}mol/L RA and without or with ($2x10^{-11}$mol/L) T3. The epithelia were fixed, sectioned and stained with Hematoxylin and Eosin (H&E) or reacted with antibodies AKH-1, 5C10, AE8 and KS19.1 to detect expression of proFG/FG, K1, K13 and K19, respectively. Dotted lines mark basal epithelial borders and bar is 100μm.

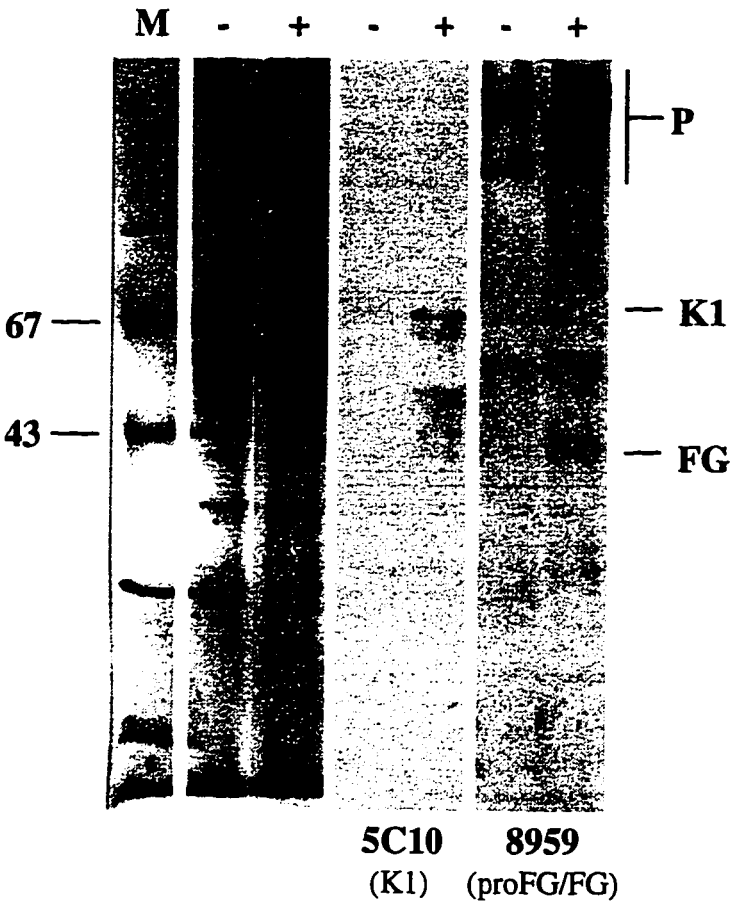

Figure 5.4: Intraexperimental confirmation of the T3 mediated decrease of the apparent RA sensitivity of gingival keratinocytes. Epithelia grown in parallel to those shown in Figure 5.3 were used for protein extraction and immunoblotting as described in Chapter II. Shown are cell extracts from keratinocytes grown at 10^{-9}mol/L RA in the presence ($2x10^{-11}$mol/L) or the absence of T3, indicated as + or - above the gel and the blots. The first panel is a Coomassie Blue stained gel, the other two panels are duplicate gels that were blotted and reacted to antibodies as marked below each blot. The first lane in the first panel shows size markers (M), two of which are marked on the left in kiloDalton. The proteins detected by the antibodies are indicated on the right; P = profilaggrin, K1 = keratin 1 and FG = filaggrin.

Several subsequent experiments aimed to substantiate the antagonistic effect of T3 on the RA induced inhibition of cornification failed to do so (data not shown). Out of a total of 12 experiments, only four experiments showed data supporting the RA/T3 antagonism, while eight experiments showed no or little effect. One explanation for the inconsistent T3 effects may have been the low T3 concentration used ($2x10^{-11}$mol/L). However, when the T3 concentration was increased 10-fold to $2x10^{-10}$mol/L in two separate experiments (these were part of the total of 12 experiments) the keratinocyte differentiation and hence the apparent RA sensitivity remained unchanged. Synergism between the two hormones was not observed in any of the above experiments.

4. Discussion and conclusions:

The experiments described in this chapter focused on the possible interaction between RA and T3 in their effects on epithelial differentiation. It was shown that there was a tendency to antagonism between the two hormones, where RA inhibited oral epithelial differentiation and T3 attenuated this inhibition. However, this T3 effect was not consistently seen throughout these experiments and even within separate experiments was the T3 antagonism inconsistent, such that effects of T3 were seen at a certain RA concentration, but not at another. It seemed like T3 was effective only during a certain, and seemingly narrow, window of opportunity. The factors governing this window of opportunity are currently unknown. In another system, namely in Xenopus laevis, T3 plays an important role in skin differentiation only during a short period of time of the amphibian metamorphosis (French et al, 1994). Whether this window of opportunity can be related to the phenomenon observed in this study is unclear.

Because the above experiments were not part of the original aim of this thesis project, they were not pursued to complete resolution. However, at the time of optimizing the organotypic raft culture for growth of oral epithelial cells, it was important to examine the RA/T3 interactions, as they may be part of a complex

hormonal cross talk (see the Background section above). Since a distinct, although inconsistent interaction was observed, T3 was left out of the growth conditions in the subsequent experiments that were performed to investigate the effects of RA on oral epithelial differentiation and a possible association with the RARs.

To date, the effects of T3 on skin are largely unknown. It is known that hypothyroidism, i.e. lack of or diminished amount of T3, is associated in high degree with coarse and dry skin reminiscent of psoriasis (Barton et al, 1991). On the other hand, topical application of an antithyroid thioureylene greatly reduces psoriatic plaques (Elias et al, 1994). Clearly, these clinical observations indicate that T3 does play a role in maintenance of epithelial morphology. Experiments in a controlled environment, such as is provided by the organotypic raft culture model, may contribute to the efforts aimed to elucidate the specific roles of T3 in epithelial biology and its possible interactions with RA.

CHAPTER VI

Influence of fibroblasts on the apparent RA sensitivity of oral keratinocytes

1. Introduction:

The results shown in this chapter are not part of the original aims of this project. However, the question of how the subepithelial tissues affect the epithelial cells is of great interest for the overall phenomenon of control and maintenance of oral epithelial differentiation. The observations regarding this question that were made during the course of the present study are a logical extension of the original goal of this project and are, therefore, included in this thesis. Although there have been previous reports about dermal/epidermal interactions in the literature (for review see Mackenzie, 1994), these experiments have as a rule consisted of various recombinations of dermis and epidermis, but the specific influences of oral fibroblasts on oral epithelial differentiation have not been investigated prior to the start of the work described in this chapter. The findings described below were in a sense "accidental", but were of such an interest that they were pursued. These fibroblast experiments were originally driven by attempts to optimize the organotypic raft culture for growth and differentiation of oral keratinocytes. The first experiments were done with collagen lattices containing GM10 fibroblasts, which originated from a fetal epidermal cell line that is commercially available (see Chapter II). The initial use of these fibroblasts was prompted by two factors: First, this was the same fibroblast cell line used by Asselineau et al (1989) in their investigation of RA effects on epidermal keratinocytes. Second, the use of these fibroblasts was one of convenience. They were available in our laboratory, ready to be used anytime. However, as attempts were made to approximate the oral keratinocyte raft culture as closely as possible to the *in vivo* differentiation of oral epithelia, the idea was born that oral fibroblasts may be more appropriate as a supporting cell type than the GM10 fibroblasts. Moreover, during

the course of the present study, several reports on the influence of fibroblasts on epithelial differentiation appeared in the literature (Wilson et al, 1992; Boisnic et al, 1993; Robert et al, 1994). Consequently, a comparison of several fibroblast types and their effect on oral keratinocyte differentiation in the organotypic raft culture model was undertaken.

2. Results:

The first experiment in this phase of the project compared GM10 fibroblasts with gingival fibroblasts (HOFs 91-14) for influences on gingival keratinocyte differentiation in culture. Figure 6.1. shows that in culture media containing 10^{-9}mol/L RA, the GM10 fibroblasts increased the apparent RA sensitivity of the keratinocytes as compared with the influence of the gingival fibroblasts. The epithelium formed in proximity to GM10 fibroblasts was a noncornified epithelium expressing K13 and K19 abundantly, but without expression of the two markers of cornification, proFG/FG and K1. In contrast, the epithelium formed with the influence of gingival fibroblasts was paracornified and expressed both proFG/FG and K1 suprabasally, but expression of K13 and K19 was virtually absent. It is not known whether the fibroblast effects are mediated via the retinoid signaling pathways, but these results are consistent with the notion that the GM10 fibroblasts allowed the keratinocytes to "see" almost 100-fold more RA as compared to the gingival fibroblasts (Figure 6.2) or alternatively, that the gingival fibroblasts protected the epithelial cells from the RA effects. In 10^{-7}mol/L RA, the epithelium formed on lattices containing gingival fibroblasts displayed a differentiation pattern very similar to the epithelium formed on lattices containing GM10 fibroblasts in 10^{-9}mol/L RA (compare to Figure 6.1). Both these epithelia were noncornified and expressed K13 and K19, but lacked proGF/FG and K1 expression. In the higher RA concentration (10^{-7}mol/L), the epithelium formed with the influence of the GM10 fibroblasts was grossly disorganized, indicating that this epithelium was "exposed" to

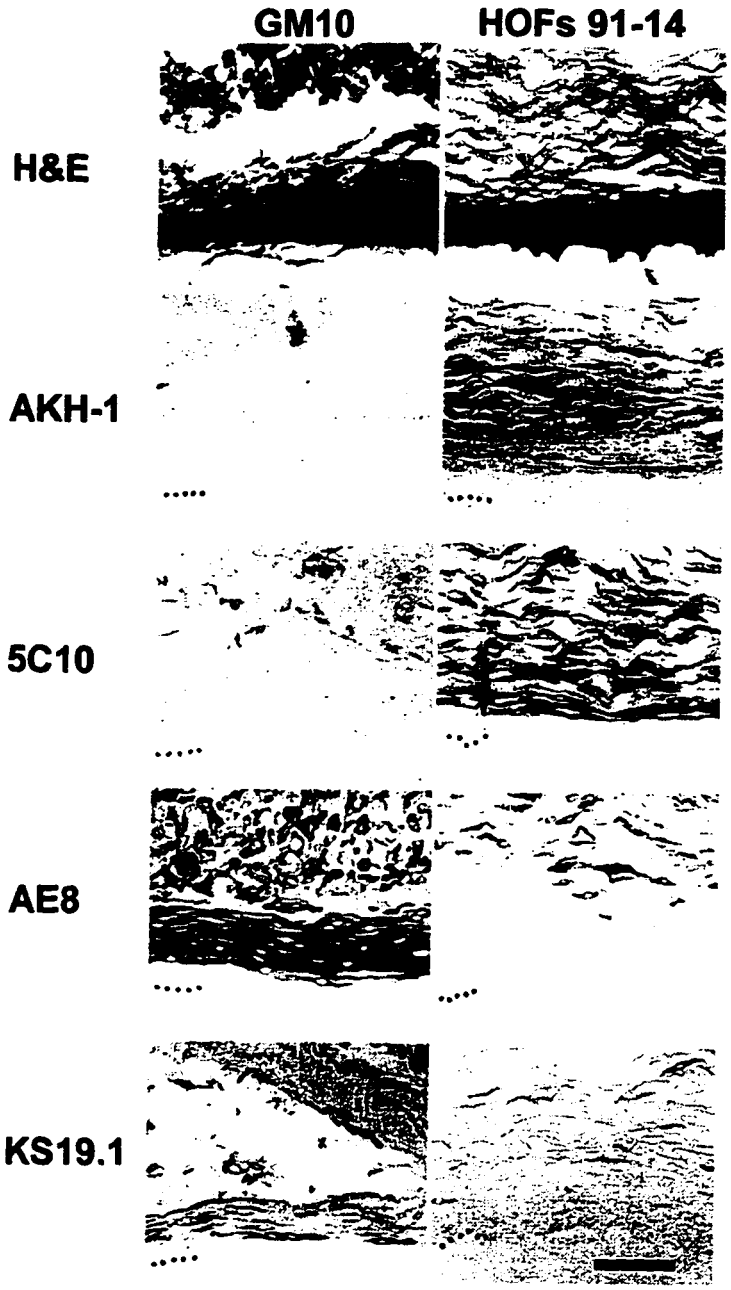

Figure 6.1: Human fetal dermal fibroblasts (GM10) induce greater RA sensitivity in gingival keratinocytes at low RA concentration than gingival fibroblasts (HOFs 91-14). Gingival keratinocytes were grown on lattices containing either GM10 fibroblasts or HOFs 91-14 in medium with 10^{-9}mol/L RA. Sections were stained with Hematoxylin and Eosin (H&E) or reacted with antibodies AKH-1, 5C10, AE8 and KS19.1 to detect expression of proFG/FG, K1, K13 and K19, respectively. Note prominent expression of K13 (AE8) and K19 (KS19.1) in the epithelium grown with GM10 fibroblasts, but expression of proFG/FG (AKH-1) and K1 (5C10) in the culture with gingival fibroblasts. Dotted lines mark the epithelial border and bar is 100μm.

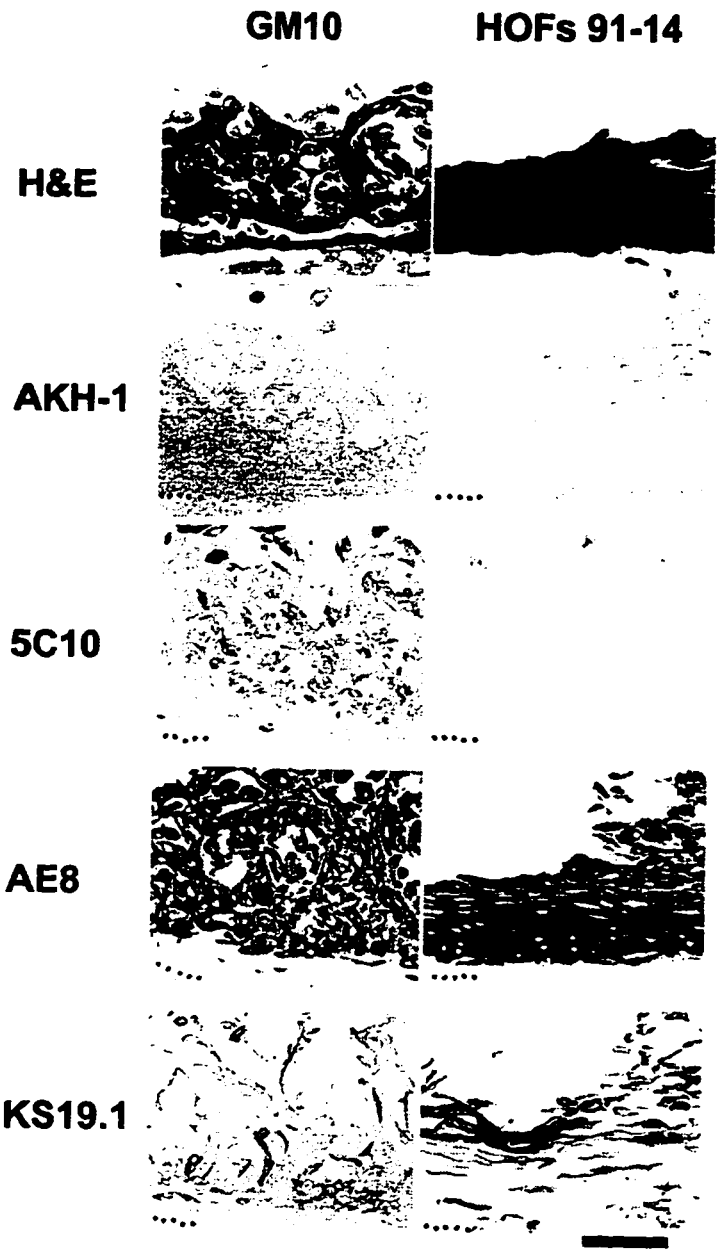

Figure 6.2: Human fetal dermal fibroblasts (GM10) induce greater RA sensitivity in gingival keratinocytes at high RA concentration than gingival fibroblasts (HOFs 91-14). Gingival keratinocytes were grown on lattices containing either GM10 fibroblasts or HOFs 91-14 in medium with 10^{-7}mol/L RA. Sections were stained with Hematoxylin and Eosin (H&E) or reacted with antibodies AKH-1, 5C10, AE8 and KS19.1 (see Figure 6.1). Note that morphology and expression of markers of differentiation of the epithelium grown with HOFs 91-14 at this RA concentration are similar to that of the epithelium grown with GM10 fibroblasts at 10^{-9}mol/L RA (see Figure 6.1). Dotted lines mark the epithelial border and bar is 100μm.

toxic levels of RA. A similar difference in the apparent RA sensitivity of the gingival keratinocytes in response to influences of the two fibroblast types was also seen in two other RA concentrations; in the control medium conditions, corresponding to approximately 10^{-8}mol/L RA and in the absence of RA (data not shown).

Another comparison was done between adult dermal fibroblasts (J.G. fibroblasts - a generous gift from Dr. Philip Fleckman) and the gingival fibroblasts (HOFs 91-14) which also originated from adult tissues. The results from this comparison clearly showed the importance of the underlying fibroblasts on terminal differentiation of epithelia (Figure 6.3), but the influence on keratinocyte RA sensitivity was less clear. In the absence of RA, gingival keratinocytes formed cornified epithelia as judged by expression of markers with both fibroblast types. However, the morphologies of the two epithelia were remarkably different. Gingival keratinocytes cultured with the gingival fibroblasts formed an epithelium resembling gingival epithelia. This epithelium was paracornified, containing numerous nuclei in the cornifying cell layers, and lacked a distinct granular cell layer. In contrast, when grown with the dermal fibroblasts, these keratinocytes formed an epithelium resembling epidermis. Although, this epithelium was also paracornified, the number of nuclei in the upper cell layers were much reduced, the epithelium was significantly thinner and did contain a distinct granular cell layer. These observations indicated that other signaling pathways than the retinoid signals may be influenced by the fibroblasts.

The effects of the fibroblasts on RA sensitive keratinocyte differentiation were more clearly evident in the high RA conditions (10^{-7}mol/L RA, Figure 6.4). Although, both epithelia formed in these conditions expressed markers of noncornified differentiation, there was a clear difference in morphology indicative of a difference in response to RA. The gingival keratinocytes formed an epithelium with a noncornified-like morphology when influenced by the gingival fibroblasts, but formed a disorganized epithelium resembling the epithelia exposed to toxic levels of RA (see chapter IV) when influenced by the dermal fibroblasts. Additionally, immunoblots showed that in the

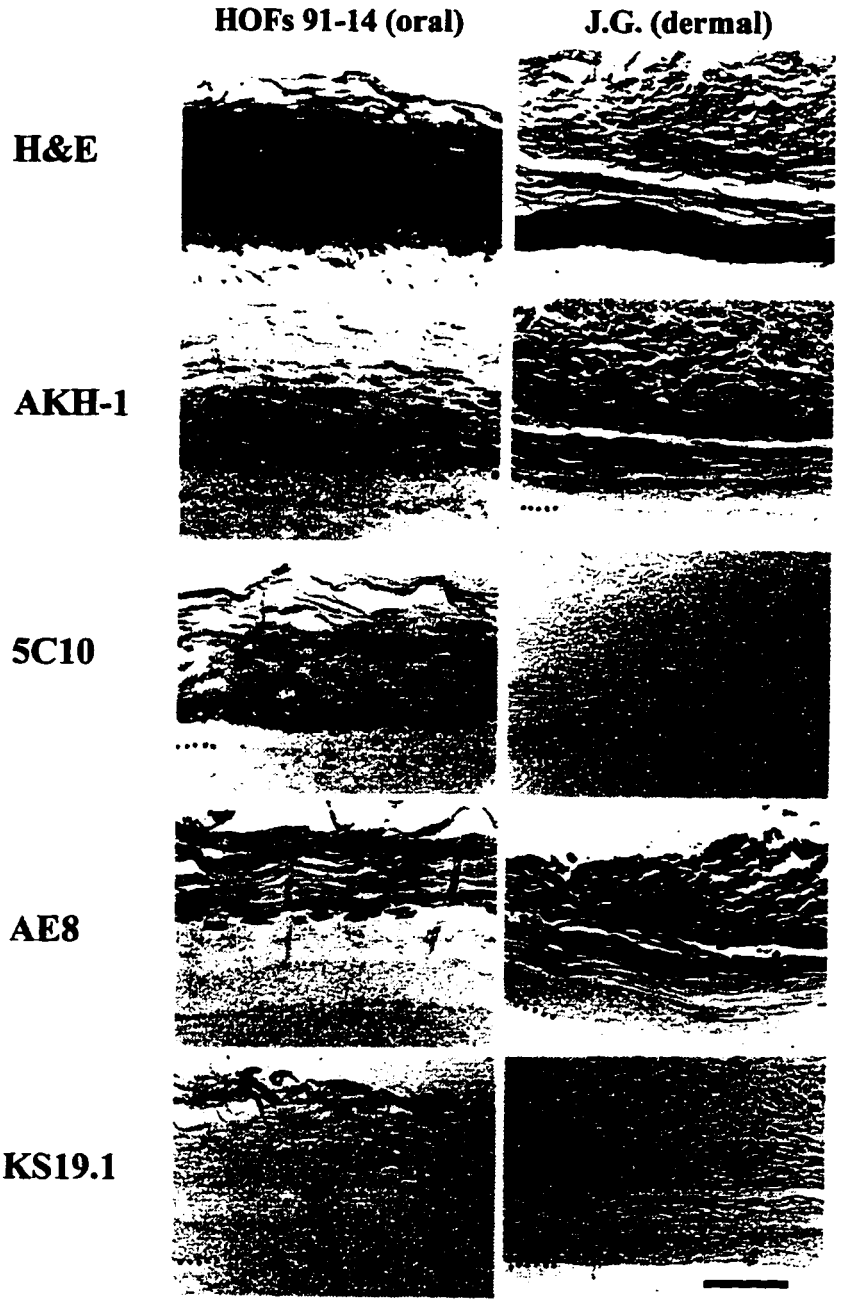

Figure 6.3: Gingival fibroblasts (HOFs 91-14) and adult dermal fibroblasts (J.G.) induce different differentiation patterns in gingival keratinocytes in the absence of RA. Epithelial morphology and expression of markers of differentiation in gingival keratinocytes grown on lattices containing either HOFs 91-14 or J.G. fibroblasts in RA free conditions were assessed by Hematoxylin and Eosin staining (H&E) and by probing with antibodies AKH-1, 5C10, AE8 and KS19.1. These antibodies detect proFG/FG, K1, K13 and K19, respectively. Note that although there are only minor differences in expression of the four markers, the morphologies of the two epithelia are markedly different. Dotted lines mark epithelial borders and bar is 100μm.

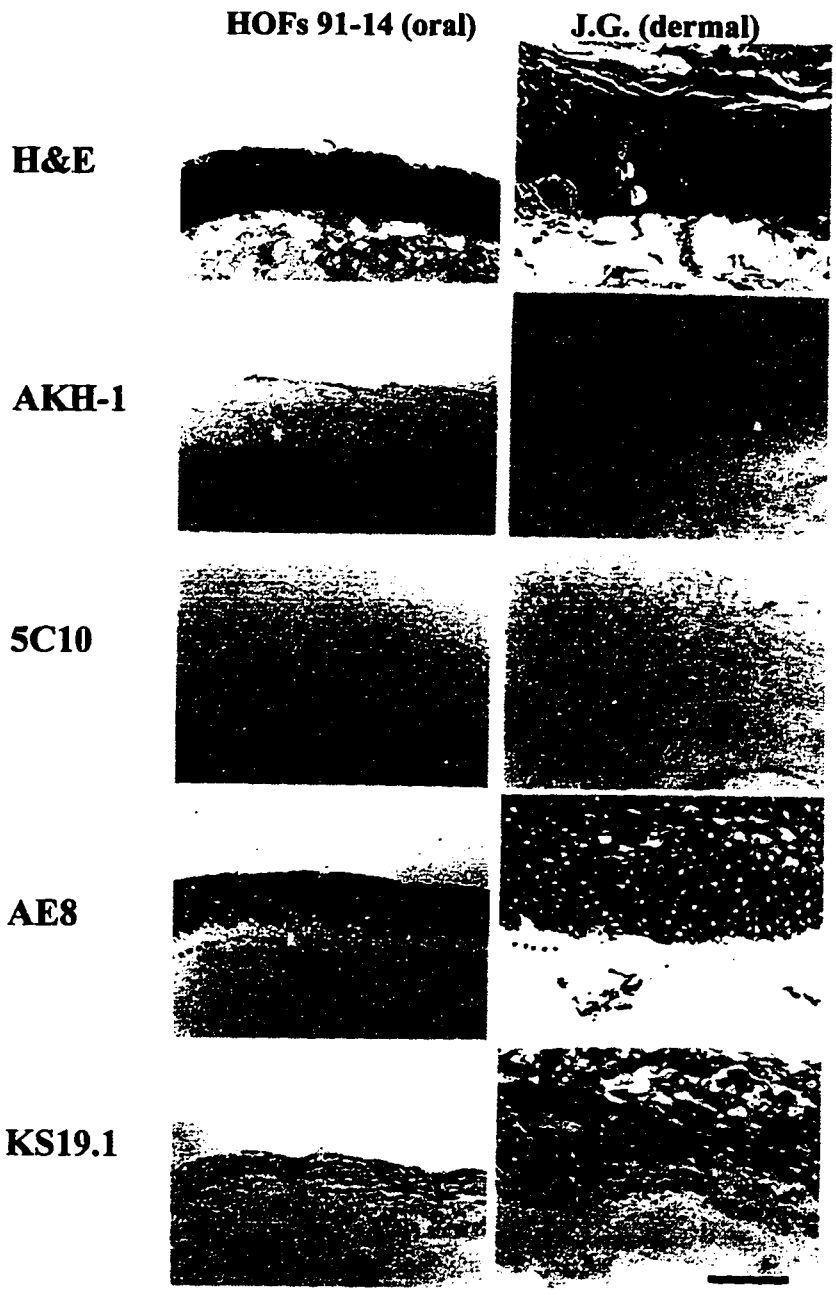

Figure 6.4: Gingival fibroblasts (HOFs 91-14) and adult dermal fibroblasts (J.G.) induce different differentiation patterns in gingival keratinocytes in the presence of 10^{-7}mol/L RA. Epithelial morphology and expression of markers of differentiation in gingival keratinocytes grown on lattices containing either HOFs 91-14 or J.G. fibroblasts in 10^{-7}mol/L RA were assessed by Hematoxylin and Eosin staining (H&E) and by probing with antibodies AKH-1, 5C10, AE8 and KS19.1. These antibodies detect proFG/FG, K1, K13 and K19, respectively. Dotted lines mark epithelial borders and bar is 100μm.

epithelium supported by the dermal fibroblasts, more K19 and less K1 were expressed (Figure 6.5). These observations are consistent with an increased RA sensitivity of the epithelial cells under these conditions.

A third comparison of different fibroblast types was done with subepithelial fibroblasts from cornified (HOFs 92-21) and from noncornified (HOFs 92-22) oral mucosa (Figure 6.6). Keratinocytes for this experiment originated from a cornified oral epithelium resembling the covering of hard palate. In conditions of high RA concentration (10^{-7}mol/L), the epithelium formed on lattices containing HOFs 92-21 (from a cornified region, C) displayed an orderly stratified, noncornified morphology. In contrast, the epithelium formed on lattices containing HOFs 92-22 (from a noncornified region, N) exhibited the disorganized morphology indicative of toxic levels of RA (Figure 6.7). At a lower RA concentration (10^{-9}mol/L), both epithelia formed were paracornified. However, the epithelium influenced by HOFs 92-22 (N) retained more of the nuclei in the upper cell layers and displayed a considerably less well defined granular cell layer. The four markers of differentiation showed no major differences between the two fibroblasts conditions at both RA concentration in the immunohistochemical analysis (data not shown), but immunoblots (Figure 6.8) showed a decrease in K1 and proFG/FG expression in the epithelia grown with HOFs 92-22 (N) as compared with epithelia grown with HOFs 92-21 (C). Together, these data show a consistent, although small difference in the effects of two oral fibroblast types on the RA sensitivity of oral keratinocytes. Interestingly, the two fibroblast types in these organotypic culture experiments mimic the influences expected to be exerted on the keratinocytes by each fibroblast type *in vivo*: That is, fibroblasts from a cornified region supported more extensive cornification of the epithelia than did fibroblasts from a noncornified region.

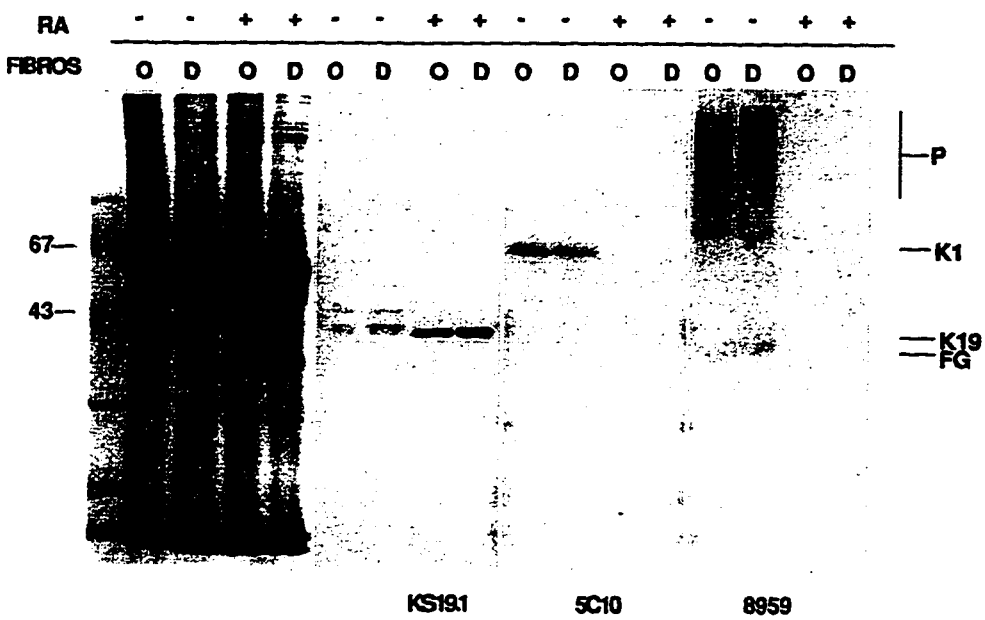

Figure 6.5: Adult dermal fibroblasts and gingival fibroblasts differentially affect expression of RA sensitive markers of differentiation in oral keratinocytes. Oral keratinocytes were cultured on lattices containing either adult dermal fibroblasts (D) or gingival fibroblasts (O)(same fibroblast strains as shown in Figures 6.3 and 6.4) in RA free conditions (-) or in media with 10^{-7}mol/L RA (+). Protein extracts from these cultures were separated on a 7.5% to 12.5% gradient sodium dodecyl sulfate gel, transferred to nitrocellulose and probed with antibodies as indicated (KS19.1 is specific for K19, 5C10 for K1 and 8959 for proFG/FG). The first panel shows a duplicate gel stained with Coomassie Blue. Molecular markers (kDa) are indicated on the left and proteins of interest on the right. P = proFG.

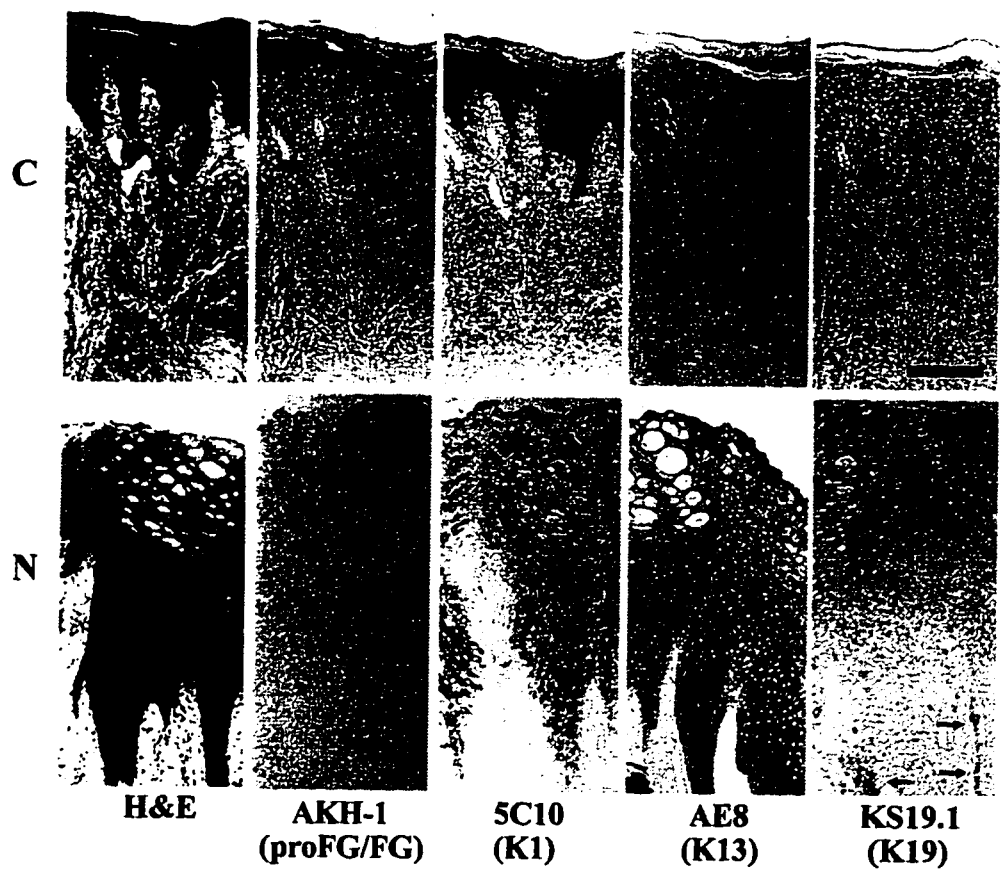

Figure 6.6: HOFs 92-21 originate from a cornified oral mucosa (C) and HOFs 92-22 from a noncornified oral mucosa (N). A representative piece of each tissue used for explant fibroblast cultures was analyzed for epithelial differentiation by assessment of morphology (H&E) and expression of markers of differentiation using antibodies AKH-1, 5C10, AE8 and KS19.1. These antibodies detect proFG/FG and specific keratins as indicated. Arrows show basal cell staining for K19. Bar is 100μm.

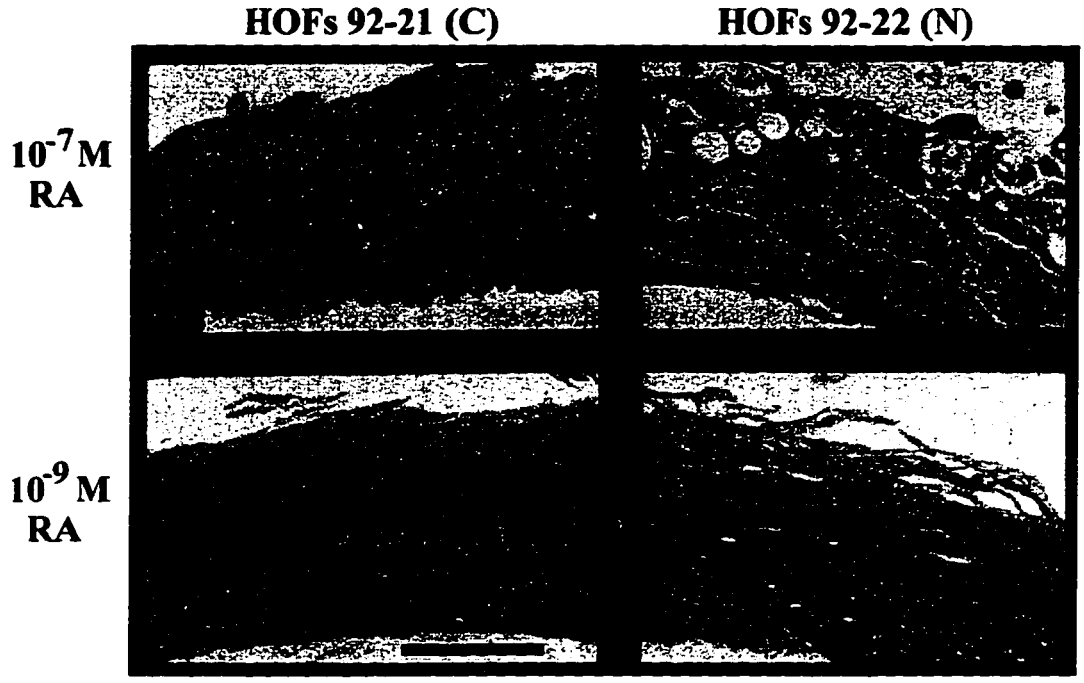

Figure 6.7: Fibroblasts from different regions of the oral cavity differentially affect morphology of gingival keratinocytes grown in the organotypic cultures. Keratinocytes were cultured on lattices containing fibroblasts from either cornified (C) or noncornified (N) oral mucosa (see Figure 6.6) in media with delipidized serum supplemented with 10^{-7} or 10^{-9}mol/L RA. Morphology was assessed by Hematoxylin and Eosin staining. Note the greater sensitivity of the keratinocytes to RA when cultured with fibroblasts from a noncornified region. This is seen as disruption of orderly stratification in the high RA concentration and as absence of a granular layer in the low RA concentration. Bar is 100μm.

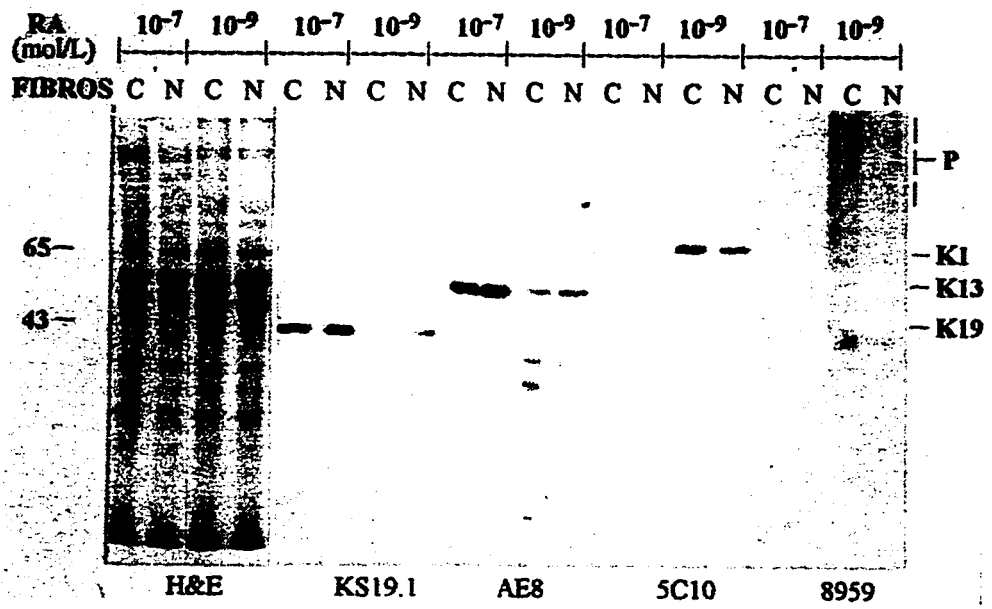

Figure 6.8: Fibroblasts from different regions of the oral cavity differentially influence expression of RA sensitive markers of keratinocyte differentiation. Oral keratinocytes were cultured on lattices containing either fibroblasts from cornified (C) or noncornified (N) oral mucosa (see Figure 6.6) in media with 10^{-7} or 10^{-9}mol/L RA. Protein extracts from these cultures were separated on a 7.5% to 12.5% gradient sodium dodecyl sulfate gel, transferred to nitrocellulose and probed with antibodies as indicated (KS19.1 is specific for K19, AE8 for K13, 5C10 for K1 and 8959 for proFG/FG). RA concentrations and coculture of fibroblasts are indicated on the top. The first panel shows a duplicate gel stained with Coomassie Blue. Molecular markers (kDa) are indicated on the left and proteins of interest on the right. P = proFG.

3. Summary and conclusion:

Experiments described in this chapter showed that subepithelial fibroblasts have an influence over the differentiation pathway taken by oral keratinocytes. Since the fibroblast effects resembled many of the effects exerted by RA, it may be hypothesized that these effects are mediated by RA signaling pathways, e.g. by changing the RA sensitivity of the epithelial cells. It was further shown that the fibroblast influences on keratinocyte differentiation in the organotypic culture largely follow the presumed communication between the two cell types occurring *in vivo*. Thus, fibroblasts from a noncornified oral region induced oral keratinocytes to form a less cornified epithelium than fibroblasts from a cornified region. The last conclusion has, however, two major caveats: First, it is not known how much of the difference seen between the two oral fibroblast types was contributed by interindividual differences, since the two fibroblast types originated from two separate patients. Nevertheless, this argument can go both ways and it is conceivable that if the two fibroblast types originated from the same patient, that the differences would have been even larger than the ones observed. Second, attempts to replicate these results have not been successful (see Chapter VII). This discrepancy is most likely dependent on the difficulty of accurately assessing the origin of the cells and this issue will be further explored in the discussion (Chapter IX). Regardless of these problems, an influence of subepithelial fibroblasts on oral keratinocyte differentiation has been convincingly shown in the series of experiments presented above and has generated a number of interesting questions about the mechanisms of this fibroblast/keratinocyte crosstalk. These fibroblast/keratinocyte interactions in a cell culture system are consistent with earlier findings from tissue recombination experiments (Mackenzie and Hill, 1984; Mackenzie, 1994, review). However, the cell culture system permits future investigation of molecular mechanisms involved in these interactions.

CHAPTER VII

Association between RAR expression and oral epithelial differentiation

1. Introduction:

Having established that retinoic acid (RA) has a profound effect on oral epithelial differentiation in the organotypic raft culture model (Chapter IV), the next major aim of this project was to investigate any possible relationships between certain patterns of epithelial differentiation and expression of the three retinoic acid receptor (RAR) subtypes. The underlying hypothesis for these efforts was that the regional variation in oral epithelial differentiation is mediated by differential expression of the RARs (see Chapter I).

In the experiments described below, oral epithelial differentiation in the organotypic raft cultures was manipulated in two ways; by use of various RA concentrations in the media alone (Chapter IV) or by use of RA and coculture with different fibroblast types together (Chapter VI). Several techniques, such as Northern blotting and S1-nuclease protection assay, were tried throughout the course of this project to quantify the low abundance messages of the three RARs. For a variety of reasons, these attempts were all unsuccessful and most likely this lack of success was due to the high sensitivity requirement of the chosen technique to detect the RARs. Finally, success was achieved by adapting a reverse transcription/polymerase chain reaction (RT/PCR) approach, which produced meaningful semi-quantitative data of RAR expression in oral keratinocytes.

2. Preliminary RAR quantification:

The initial assessment of keratinocyte RAR expression was done in two pilot experiments. These two experiments (92-17 and 93-5) were not numerically quantified as described in Chapter II, but instead, relative amounts of the amplified RAR products were visually assessed from thickness and intensity of specific bands on ethidium bromide stained acrylamide gels. This initial phase of the RAR quantification served mainly as a learning ground for further development and improvement of the technique. Nevertheless, these experiments produced results in regard to RARβ expression that were verified and extended in subsequent and more definitive experiments, namely that RARβ expression was increased in epithelia with lesser degree of cornification.

Epithelial differentiation in the first pilot experiment (92-17) was manipulated by varying the RA exposure. In absence of RA in the culture media, the gingival keratinocytes grown on lattices containing oral fibroblasts (HOFs 91-14) formed a paracornified epithelium. When RA was added to a concentration of 10^{-7}mol/L, these keratinocytes formed a non-cornified like, but well stratified epithelium (data not shown). Thus, this experiment reproduced previous experiments investigating the RA effects on gingival keratinocytes (see Figures 6.1 and 6.2). In the other preliminary experiment (93-5), epithelial differentiation was manipulated by both RA and oral fibroblasts. The keratinocytes used in this experiment also originated from a gingival epithelium and they were cocultured with HOFs 92-21 (originating from a cornified region) and HOFs 92-22 (originating from a noncornified region). In this experiment, the results from previous experiments (see Figure 6.7) were also largely reproduced (data not shown). Thus, in absence of RA, in both fibroblast conditions the epithelia formed were paracornified. However, with the influence of fibroblasts from a cornified region (HOFs 92-21), the epithelium showed a clearly distinct granular cell layer, which was absent in cocultures with fibroblasts from a noncornified region (HOFs 92-22). In presence of 10^{-7}mol/L RA, both epithelia lacked signs of cornification. While the

epithelium formed under the influence of HOFs 92-21 showed an orderly stratification, HOFs 92-22 induced a disorganized morphology indicative of a higher degree of RA sensitivity in these epithelial cells. The relative cornification of each epithelium is shown in Table 7.1.

Total RNA was extracted from epithelia cultured in parallel to those used for histological examination. This RNA was then subjected to the semi-quantitative RT/PCR experiments as discussed above. Data from these two experiments are shown in Table 7.1. Although there was a fair amount of variation in these data, one thing stood out. In each experiment, RARβ expression was increased as the differentiation of the epithelia switched from a cornified to a noncornified pattern. Additionally, the RARβ increase was actually larger than shown, because of the use of β-actin as internal control (see Table 7.2, below). Thus, these preliminary experiments indicated that RARβ expression was increased as oral epithelial cornification was decreased. No definite conclusions could be drawn regarding expression of RARα and RARγ from these preliminary experiments.

3. Choice of controls for the RT/PCR quantification of RAR expression:

The semi-quantitative determination of RAR message by use of PCR was based on the assumption that the RT reactions, which convert mRNA to the cDNA that was subsequently used as template for the PCRs, correctly mirror the relationship between a constitutively expressed control message and the expression of RARs in the different experimental conditions within each experiment. In the pilot experiments described above, β-actin was used as such a constitutively expressed (housekeeping) gene. This choice was made, because this gene product has been routinely used for this purpose by other investigators (Redfern and Todd, 1992; Wan et al, 1992; Sharpe et al, 1994, Smith et al, 1995). However, several reports in the literature show that expression of actin and

Table 7.1: Preliminary evaluation of RAR expression in cultured epithelia.

RA conditions:		0 mol/L	10^{-7}mol/L
Cornification:[a]		++	--
PCR 1[b,c]	control (β-actin)	+	+
	RARα	ND[d]	ND
	RARβ	+/-	+
	RARγ	++	+

Fibroblasts:		92-21 (C)	92-22 (N)	92-21 (C)	92-22 (N)
RA conditions:		0 mol/L	0 mol/L	10^{-7}mol/L	10^{-7}mol/L
Cornification:[a]		++	+	-	--
PCR 1[c]	control (β-actin)	+	++	++	+++
	RARα	ND	ND	ND	ND
	RARβ	+/-	+/-	+	+++
	RARγ	+	+	++	+++
PCR 2	control (β-actin)	+	+	++	++
	RARα	ND	ND	ND	ND
	RARβ	+/-	+/-	+	+++
	RARγ	+	+	+++	+++
PCR 3	control (β-actin)	+	+	+	+
	RARα	+	+	+/-	++
	RARβ	+/-	+/-	+	++
	RARγ	+	+	+	+

[a] Relative degree of cornification was estimated by immunohistochemical data and morphology of the cultured epithelia using ++ as most cornified and -- as least cornified.

Table 7.1: (Continued).

[b] Quantification of the mRNAs was done as described in Chapter II, except that β-actin was used as loading control and the PCRs were not spiked with radiolabelled dATP. The amount of the PCR products was visually analyzed from thickness and intensity of bands on ethidium bromide stained gels and relative degree of expression of each product was estimated as +/- for least expression and +++ for most abundant expression.
[c] Results from experiment 92-17.
[d] ND = not done.
[e] Results from experiment 93-5.

the cellular distribution of the actin skeleton is dependent on the state of differentiation of epithelial cells (Watt et al, 1993, Migdal et al, 1995). The use of β-actin as a RA- and differentiation-independent message was therefore questioned and its expression was compared to two other commonly used "housekeeping" messages, namely glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and the acidic ribosomal phosphoprotein (PO) (Laborda, 1991).

This comparison was done in a lifted culture experiment, in which the resulting epithelia showed variations in differentiation as in the previous work. This variation, ranging from a paracornified to a noncornified epithelium with disorderly stratification (Figure 7.1), was achieved by culturing the oral keratinocytes in absence (0 mol/L) and presence (10^{-7}mol/L) of RA and with two different oral fibroblast strains (HOFs 91-14 (from cornified oral mucosa) and HOFs 92-22 (from noncornified oral mucosa)). When the RT-cDNA loadings from the four experimental conditions were normalized to G3PDH expression, PO expression remained relatively constant, while β-actin expression increased with a decrease in epithelial cornification (Table 7.2).

The same comparison was subsequently performed with RNA extracts from epithelia taken from a cornified oral region (hard palate) and from a noncornified oral region (buccal vestibule) from the same subject. As shown in Figure 7.2, the palatal mucosa is covered by an orthocornified epithelium expressing proFG/FG in the granular layer, but not expressing K13. In contrast, the mucosa taken from the buccal vestibule is covered by a noncornified epithelium, which displays the reverse expression of these two markers. Because these two tissues were removed from the subject under controlled conditions, this is the only instance in this study in which the purity of the determined differentiation pattern of each tissue can be clearly documented (for further discussion see Chapter IX).

In this comparison, when the cDNA (template) loadings were adjusted for G3PDH, PO expression did not vary significantly in the two epithelia, while β-actin expression was approximately 9-fold higher in the noncornified epithelium (Table 7.3).

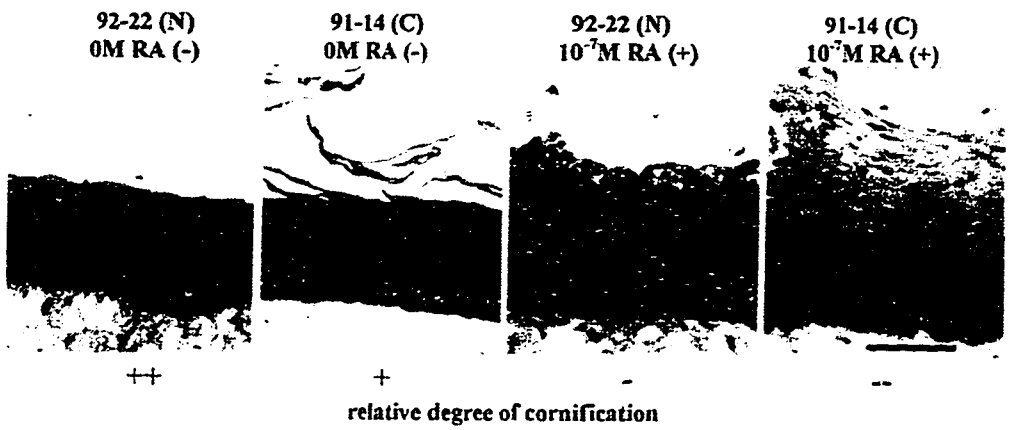

Figure 7.1: RA inhibits epithelial cornification and fibroblasts modify the apparent RA effects in organotypic cultures. Epithelia formed by gingival keratinocytes which were grown in organotypic cultures in 0 mol/L RA (-) or 10^{-7}mol/L RA (+) with two different strains of oral fibroblasts (92-22 or 91-14), were fixed, sectioned and stained with Hematoxylin and Eosin for analysis of morphology. Fibroblast strain 92-22 originated from a noncornified oral mucosa (N), while strain 91-14 was from a cornified oral region (C). Note that these two fibroblast strains were from two separate patients. Relative epithelial cornification is marked as ++ for the most cornified and as -- for the least cornified epithelium. Bar is 100μm.

Table 7.2: Comparison of expression of potential control messages in organotypic raft cultures that display variation in oral epithelial differentiation.

Fibroblast strain:	HOFs 92-22	HOFs 91-14	HOFs 92-22	HOFs 91-14
RA conditions:	-RA	-RA	+RA	+RA
Cornification:[a]	++	+	-	--
G3PDH	100[b]	105	100	88
PO	100	90	114	98
β-actin	100	167	560	430

[a] Relative degree of cornification was estimated by immunohistochemical data and morphology of the cultured epithelia using ++ as most cornified and -- as least cornified (see Figure 7.1).
[b] Expression of each mRNA was numerically quantified by incorporation of radiolabel in the PCR assays with subsequent scanning by phosphorimaging (see Chapter II). The values are shown as percent expression of the expression in the most cornified sample, which was set at 100%. Note that while G3PDH and PO expression remained essentially unchanged throughout the four experimental conditions, b-actin expression was markedly increased as the degree of epithelial cornification was decreased.

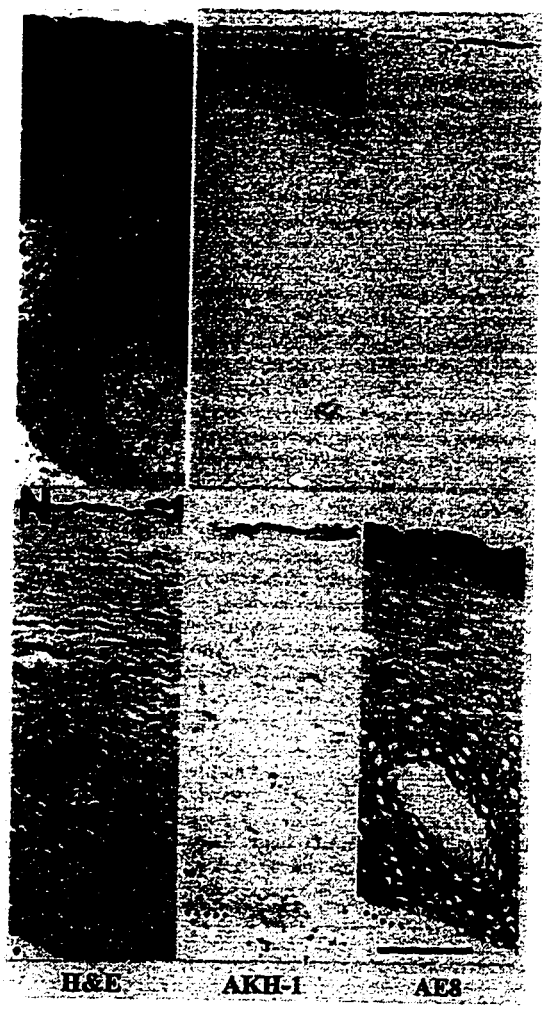

Figure 7.2: Oral epithelia from hard palate and from buccal vestibule differ in degree of cornification. Sections from a palatal and a buccal biopsy taken from one subject were analyzed for morphology by Hematoxylin and Eosin staining (H&E) and for expression of markers of differentiation by immunohistochemistry. Shown markers are proFG/FG detected with antibody AKH-1 and K13 detected with antibody AE8. C = cornified epithelium, N = noncornified epithelium. Dotted lines indicate the border between basal keratinocytes and underlying connective tissue. Bar is 100μm.

Table 7.3: Comparison of expression of potential control messages in a cornified[a] and a noncornified[b] oral epithelium.

Tissue origin:	Hard palate	Buccal fold
Degree of cornification:[c]	++	--
PO	100[d]	83
G3PDH	100	149
β-actin	100	886

[a] The cornified epithelium came from a palatal biopsy specimen (see Figure 7.2).
[b] The noncornified epithelium came from a buccal biopsy specimen (see Figure 7.2).
[c] Relative degree of cornification was estimated by immunohistochemical data and morphology of the two tissues using ++ as most cornified and -- as least cornified.
[d] Expression of each mRNA was numerically quantified by incorporation of radiolabel in the PCR assays with subsequent scanning by phosphorimaging (see Chapter II). The values are shown as percent expression of the expression in the most cornified sample, which was set at 100%. Note that while G3PDH and PO expressions were similar in the two epithelia, β-actin expression was markedly higher in the noncornified tissue.

Additionally, expression of the transferrin receptor (Schneider et al, 1984) was analyzed as a potential control message. This analysis was undertaken for two reasons: First, the transferrin receptor was marketed by Clontech (Palo Alto, CA; 95/96 catalog, p. 43) as a control gene product for RT-PCRs. Second, the transferrin receptor message is expressed at low levels in most cell types and would therefore be an ideal transcriptional control for the low abundance RAR messages. In this analysis of the lifted cultures, the transferrin receptor was barely expressed in the cornified epithelia, but was strongly induced in the noncornified, RA treated epithelia when compared to the expression of G3PDH mRNA (data not shown). In this experiment, the induction of the transferrin receptor mRNA was not numerically quantified and was therefore not included in the above table (Table 7.2). A numerical quantification of this message was, however, done in the two oral epithelia (cornified and noncornified, see Figure 7.2) and again the transferrin receptor message was significantly higher (approximately 17-fold higher) in the noncornified epithelium when the RT-cDNA loads into the PCRs were adjusted for G3PDH and PO. In fact, the difference in transferrin receptor expression in the two epithelia was even greater than the difference in β-actin expression. Indeed, several reports in the literature indicate that transferrin receptor expression is correlated with the proliferation rate of most cell types, including the keratinocyte (Oliver et al, 1991; Kennedy et al, 1992; Darcissac et al, 1996). Finally, it should be mentioned that Clontech suggests the use of transferrin receptor as a RT-PCR control rather than a transcriptional control or housekeeping product, claiming only that its message is ubiquitously expressed. The results here support only limited use of the transferrin receptor as a control.

In summary, the above findings indicated that neither β-actin nor transferrin receptor are suitable RT-PCR controls where quantification of the message is needed and that G3PDH or PO are better suited as such controls. Although some preliminary experiments were done with β-actin as the transcriptional control, the subsequent studies used PO as control. PO was chosen rather than G3PDH purely for convenience.

4. Semi-quantitative analysis of RAR expression in cultured epithelia:

The conventional RT/PCR technique used in the pilot experiments was adapted for subsequent experiments in order to more precisely and reliably quantify the low abundance RAR messages. As described in detail in Chapter II, the quantification of the RARs was based on the assumption that the proportions between various messages remained unaltered during the RT phase of the protocol. The loading of template into the subsequent amplification phase was therefore adjusted in reference to a "house keeping" gene product as discussed above. A numerical quantification in the amplification phase was achieved by adding small amounts of radioactive dATP to the PCR mixtures. The amount of the radionucleotide incorporated into the final product became proportional to the amount of product made, thus providing information about the relative amount of the template put into the PCRs and consequently, about the relative amount of the mRNAs originally used for the RT reactions.

Using this RT/PCR technique, keratinocyte RAR expression was evaluated in respect to oral epithelial differentiation. In the first example (Figure 7.1), epithelial differentiation was manipulated by use of two RA conditions and two oral fibroblast types. This approach yielded four different patterns of epithelial differentiation, two cornified and two noncornified. These results largely reproduced results from previous experiments (compare to Figure 6.7).

Epithelial differentiation in the second example (Figure 7.3), was manipulated by use of three RA concentrations in the culture media (0 mol/L, 10^{-8}mol/L and 10^{-7}mol/L). Again, the differentiation results were largely reproduced from earlier experiments. In absence of RA, the keratinocytes formed a paracornified epithelium with a granular cell layer immediately below stratum corneum. When 10^{-8}mol/L RA was added to the culture medium, the epithelium took on a noncornified-like morphology without a granular cell layer and retained most of the cell nuclei in the upper most cell layers. Upon addition of 10^{-7}mol/L RA, the epithelium started to show various disruptions of

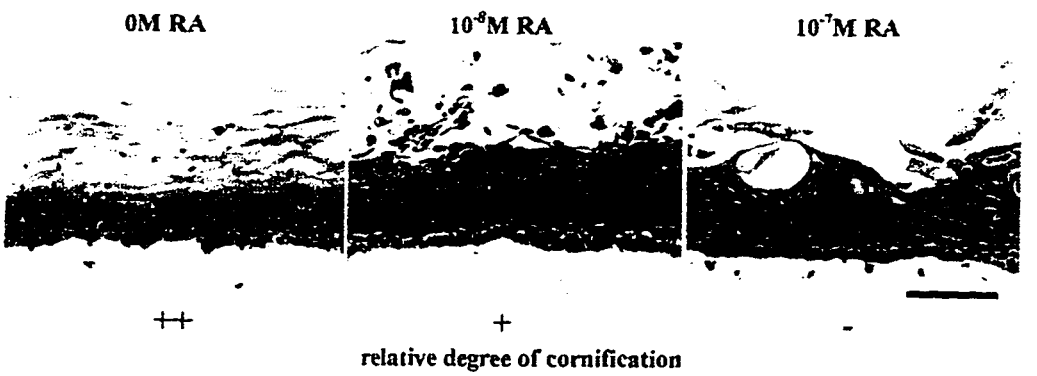

Figure 7.3: RA inhibits oral epithelial cornification in organotypic cultures. Epithelia formed by gingival keratinocytes in organotypic cultures were grown in three different RA concentrations; 0, 10^{-8} and 10^{-7}mol/L RA. The epithelia were fixed, sectioned and stained with Hematoxylin and Eosin for analysis of morphology. Relative cornification is marked as ++ for the most cornified epithelium and as - for the least cornified epithelium. Bar is 100μm.

the ordered morphology. In addition, the differences in these morphologies were mirrored in the expression of markers of differentiation (data not shown). With increasing RA concentrations there was a decrease in expression of markers of cornification (proFG/FG and K10) and an increase in markers of noncornified epithelia (K13 and K19).

Table 7.4 shows the complete set of data from the RT/PCRs performed to quantify keratinocyte RAR expression in these two experiments. These data illustrate the major finding that RARβ expression was increased in the cultured epithelia in correlation with a decrease in cornification. However, the complexity of the semi-quantitative RT/PCR results required statistical consultation (Statistical Consulting Services, Department of Biostatistics, University of Washington, Seattle, WA) to seek ways to compress the data so that major, reproducible findings could be summarized and evaluated. These data illustrate some of the peculiarities of and difficulties with the RT/PCR quantifications (see footnotes, Table 7.4). It became clear, that errors can occur at both the RT as well as the PCR steps in this technique. Thus, it was important to reproduce the quantifications with templates from repeated RT reactions. In addition, choice of the particular primer pair also turned out to be of major importance. Because of the technical complexity of the RT/PCR methodology, experiments that showed these types of errors or inconsistencies in pattern of RAR expression were considered as outlyers and were excluded from the final analysis of the data.

Figures 7.4 and 7.5 show a representative PCR autoradiogram from each of the two experiments and a graphical representation of the reproducible data from these experiments. The graphical summaries omit experiments in which control amplimers and other factors were not adequately controlled. These summaries clearly show that in both experiments, RARβ expression was greatly increased as the epithelia shifted from a cornified to a noncornified pattern of differentiation. In contrast, RARα and RARγ expressions remained practically unchanged in these epithelia. The RARβ increase

Table 7.4: Semi-quantitative evaluation of RAR expression in cultured epithelia as a function of differentiation[a].

Fibroblasts:		HOFs 92-22	HOFs 91-14	HOFs 92-22	HOFs 91-14
RA conditions:		-RA	-RA	+RA	+RA
Cornification:[a]		++	+	-	--
RT #1[d] PCR #1	c (G3PDH)[b]	100[c]	104	79	72
	RARα	100	558	164	458
	RARβ$_{105}$	100	550	367	414
	RARγ	100	631	219	533
RT #1[d] PCR #2	c (G3PDH)	100	108	76	97
	RARα	100	28345	1334	24822
	RARβ$_{105}$	100	95	482	394
	RARγ	100	753	205	739
RT #2[e] PCR #1	c (G3PDH)	100	87	173	81
	RARα	ND	ND	ND	ND
	RARβ$_{105}$	100	2	582	355
	RARγ	100	166	403	345
RT #2[e] PCR #2	c (G3PDH)	100	202	136	280
	RARα	100	536	532	944
	RARβ$_{105}$	ND	ND	ND	ND
	RARγ	100	166	437	340
RT #2 PCR #3	c (G3PDH)	100	105	100	88
	c (PO)	**100**	**90**	**114**	**98**
	RARα	**100**	**305**	**297**	**367**
	RARβ$_{105}$[f]	100	130	660	559
	RARβ$_{208}$	**100**	**31**	**2521**	**2543**
	RARγ	**100**	**168**	**319**	**329**
RT #3 PCR #1	**c (PO)**	**100**	**60**	**81**	**72**
	RARα	**100**	**115**	**261**	**ND**
	RARβ$_{208}$	**100**	**ND**	**2836**	**7002**
	RARγ	**100**	**94**	**203**	**133**

Table 7.4: (Continued).

RT #3[g] PCR #2	RARα	+	+	++	ND

RA condition:		0 mol/L	10^{-8}mol/L	10^{-7}mol/L
Degree of cornification:		++	+	--
RT #1[c] PCR #1	c (PO)	100[h]	80	30
	RARα	100	55	29
	RARβ	100	555	786
	RARγ	100	80	208
RT #1 PCR #2	**c (PO)**	**100**	**88**	**124**
	RARα	**100**	**79**	**88**
	RARβ	**100**	**1899**	**31974**
	RARγ	ND	ND	ND
RT #1 PCR #3	**c (PO)**	**100**	**111**	**141**
	RARα	**100**	**32**	**113**
	RARβ	**100**	**466**	**5602**
	RARγ	**100**	**59**	**97**
RT #2[i] PCR #1	c (PO)	+++	+++	+++
	RARα	++	++	+++
	RARβ	+/-	+	+++
	RARγ	+++	+++	+++
RT #2 PCR #2	**c (PO)**	**100**	**71**	**91**
	RARα	**100**	**99**	**183**
	RARβ	**100**	**1467**	**22565**
	RARγ	**100**	**46**	**187**

[a] Epithelial differentiation was manipulated as described in the text above and relative degree of cornification was estimated using ++ as most cornified and -- as least cornified.

[b] c = control.

[c] Data from experiment 94-4. Expression of each mRNA was numerically quantified by incorporation of radiolabel in the PCR assays with subsequent scanning by phosphorimaging (see Chapter II). The values are shown as percent expression of the expression in the most cornified sample, which was set at 100%. In experiments where numerical quantification was

Table 7.4: (Continued).

not possible, levels of expression were quantified visually as described in Table 7.1. ND = not detected. Data in boldface were used in the final interpretation (see Figure 7.4).

[d] RT excluded from final analysis. Note the large increase in RAR expression in the "+" condition. This increase was not observed in other RT experiments.

[e] PCRs excluded from final analysis, because of poorly controlled loading of internal control.

[f] PCR excluded from final analysis, because of a constant problem with contamination in the negative controls with this primer pair. In spite of that, a "significant" increase in RARβ expression was detected.

[g] PCR excluded from final analysis, because it was not numerically quantified. Note that the visual quantification reproduced the results from RT #3, PCR #1 from that experiment.

[h] Data from experiment 95-21 (for a summary of these data see Figure 7.5).

[i] PCR excluded from final analysis, because it was not numerically quantified due to technical problems. Note, however, that the visual quantification of RAR expression follows the reproducible pattern of RAR expression in that experiment.

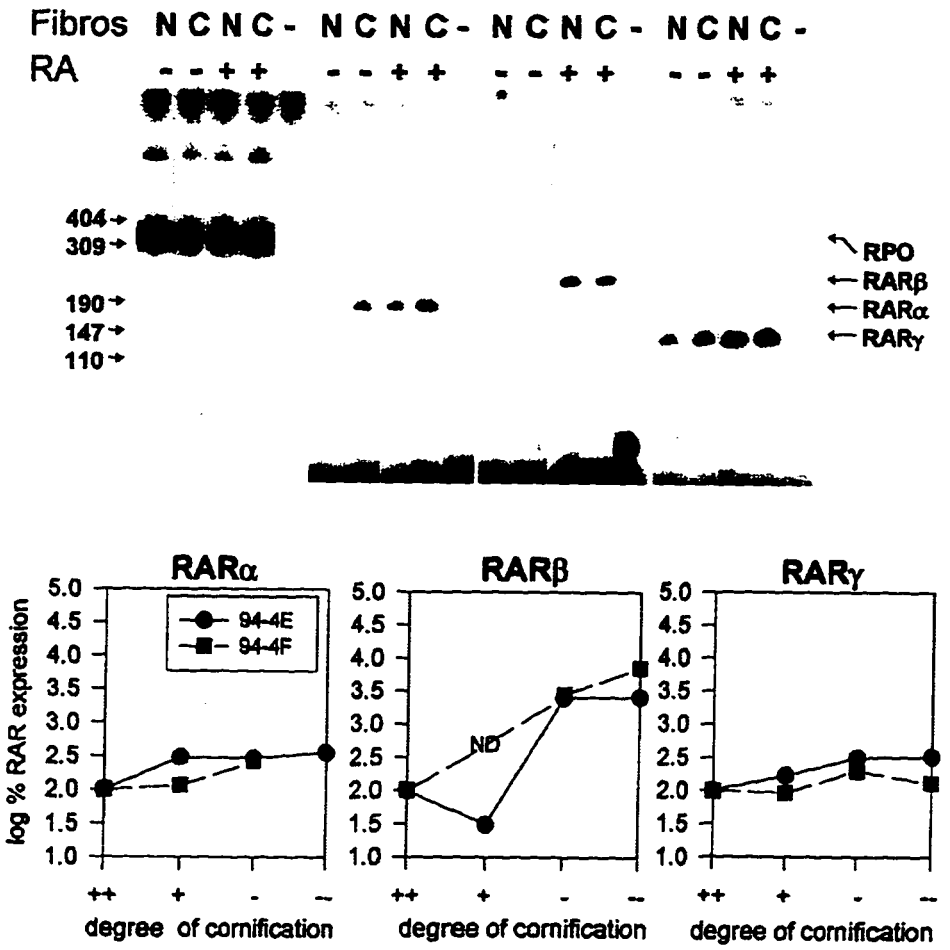

Figure 7.4. RARβ expression is inversely associated with cornification of oral epithelia grown in organotypic culture - results from experiment shown in Figure 7.1. The upper part shows an autoradiogram from a representative PCR quantification. Shown are amplifications of the control message, ribosomal phosphoprotein (RPO), and the three RAR subtypes (RARα, RARβ and RARγ). Origin of the fibroblasts and the RA concentrations used for each experimental group are indicated above each lane. The fibroblast are denoted as N (strain HOFs 92-22) and C (strain HOFs 91-14) and the RA concentrations are shown as - (0 mol/L) and + (10^{-7}mol/L). The fifth lane in each panel is a negative control amplification for each product. Marker sizes in base pairs are shown on the left. The lower part is a graphic representation of the RAR quantification including an additional PCR experiment. Each RAR subtype is plotted as log% expression, where the expression in the most cornified epithelium has been set to 100%. Data shown are from two separate RT reactions (see Table 7.4). ND = not detected.

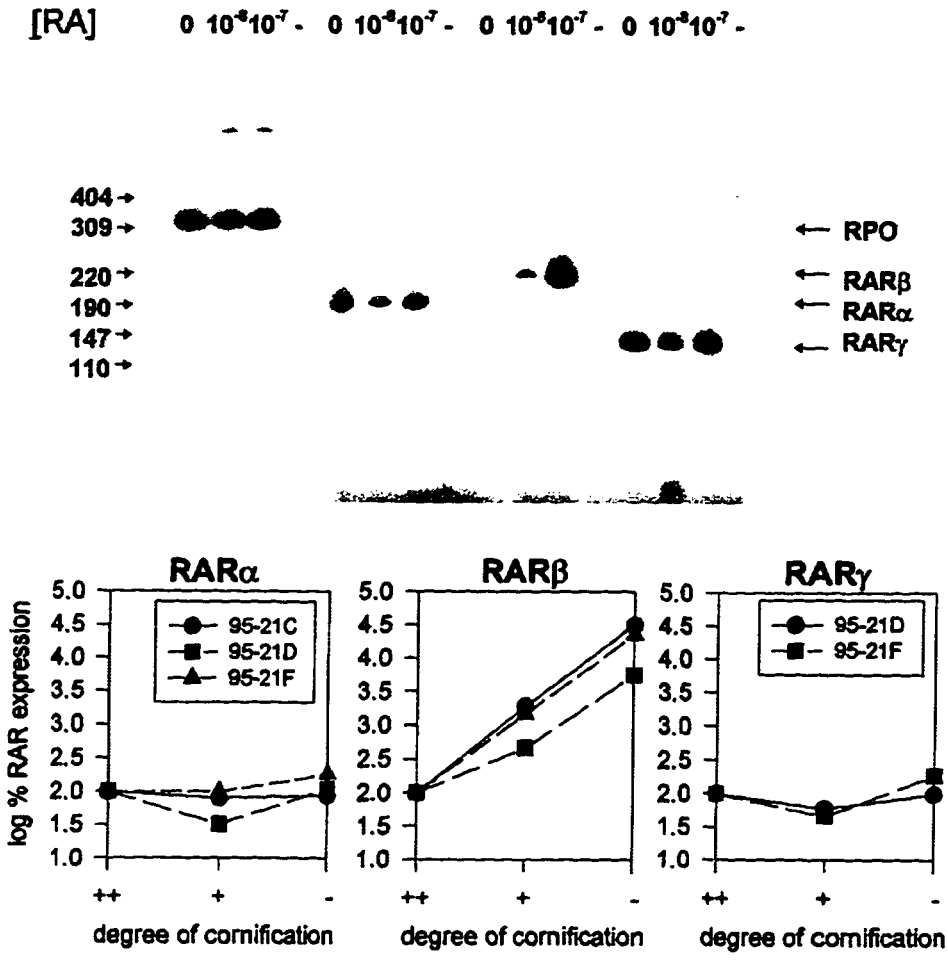

Figure 7.5: RARβ expression is inversely associated with cornification of oral epithelia grown in organotypic culture - results from experiment shown in Figure 7.3. The upper part shows an autoradiogram from a representative PCR quantification. Shown are amplifications of the control message, ribosomal phosphoprotein (RPO), and the three RAR subtypes (RARα, RARβ and RARγ). The RA concentrations for each experimental group are indicated above each lane and marker sizes in base pairs are shown on the left. The fourth lane in each panel shows a negative control amplification for each product. The lower part is a graphic representation of the RAR quantification with additional PCR experiments included. Each RAR subtype is plotted as log% expression, where the expression in the most cornified epithelium has been set to 100%. Data shown are from two separate RT reactions (see Table 7.4, experiment 95-21).

ranged between 25-fold and 320-fold. However, because of the semi-quantitative character of this technique, these numbers should not be considered as the absolute quantification of the RARβ increase. Yet, they were (and will be) useful as reference to present (and future) experiments employing this RT/PCR method.

5. Semi-quantitative analysis of *in situ* RAR expression in oral epithelia:

The final aim of this part of the project was to substantiate the RAR expression data in cultured oral epithelia by comparing *in situ* expression of the RARs in an oral cornified and an oral noncornified epithelium. For this experiment oral tissues were taken from one subject under controlled conditions to ensure that the two biopsies contained only one type of epithelium. The biopsies were taken from normal mucosa covering the palate (cornified epithelium) and from normal mucosa covering the buccal vestibule (noncornified mucosa). Epithelial differentiation of these two tissues is shown in Figure 7.2. (also see text above, Section 3 of this Chapter). The epithelia were separated from the underlying tissues as described in Chapter II, total RNA extracted and RAR expression quantified. Thus, the RNA analysis contained material from the epithelial tissues only without contamination of the underlying connective tissues. Data from three quantitative PCRs done with templates from two separate RT reactions are shown in Table 7.5. and a representative PCR autoradiogram and two of these experiments are graphed in Figure 7.6. These data clearly indicate that expression of RARα was similar in the two oral epithelia. In contrast, RARβ expression was 110 to 150-fold higher in the noncornified epithelium as compared to the cornified counterpart. It is notable that the difference in RARβ expression falls in the range of the RARβ increase observed between the cultured cornified and noncornified epithelia. Finally, RARγ expression was approximately two-fold higher in the noncornified oral epithelium. However, since this difference was below or at the sensitivity limits of the semi-quantitative RT/PCR technique, it must be viewed as normal experimental variation.

Table 7.5: Semi-quantitative evaluation of *in situ* RAR expression in cornified and noncornified oral epithelia.[a]

Tissue Origin:		Palate	Buccal fold
Degree of cornification:		++	--
RT #1 PCR #1	**c (PO)[b]**	**100[c]**	**87**
	RARα	**100**	**81**
	RARβ	**100**	**14789**
	RARγ	**100**	**219**
RT #2[d] PCR #1	c (PO)	++	++
	RARα	+	+
	RARβ	+/-	++
	RARγ	+	+
RT #2 PCR #2	**c (PO)**	**100**	**100**
	RARα	**100**	**98**
	RARβ	**100**	**11231**
	RARγ	**100**	**191**

[a] The cornified epithelium was from a palatal biopsy and the noncornified epithelium was from a buccal fold biopsy from the same subject (see Figure 7.2).

[b] c = control

[c] Expression of each mRNA was numerically quantified by incorporation of radiolabel in the PCR assays with subsequent scanning by phosphorimaging (see Chapter II). The values are shown as percent expression of the expression in the cornified sample, which was set at 100%. In experiments where numerical quantification was not possible, levels of expression were quantified visually as described in Table 7.1. Data in boldface were used in the final interpretation (see Figure 7.6).

[d] PCR excluded from final analysis due to technical difficulties with numerical quantification of the PCR products. Note, however, that the visual quantification of this experiment concurs with the results from the two other RT/PCRs.

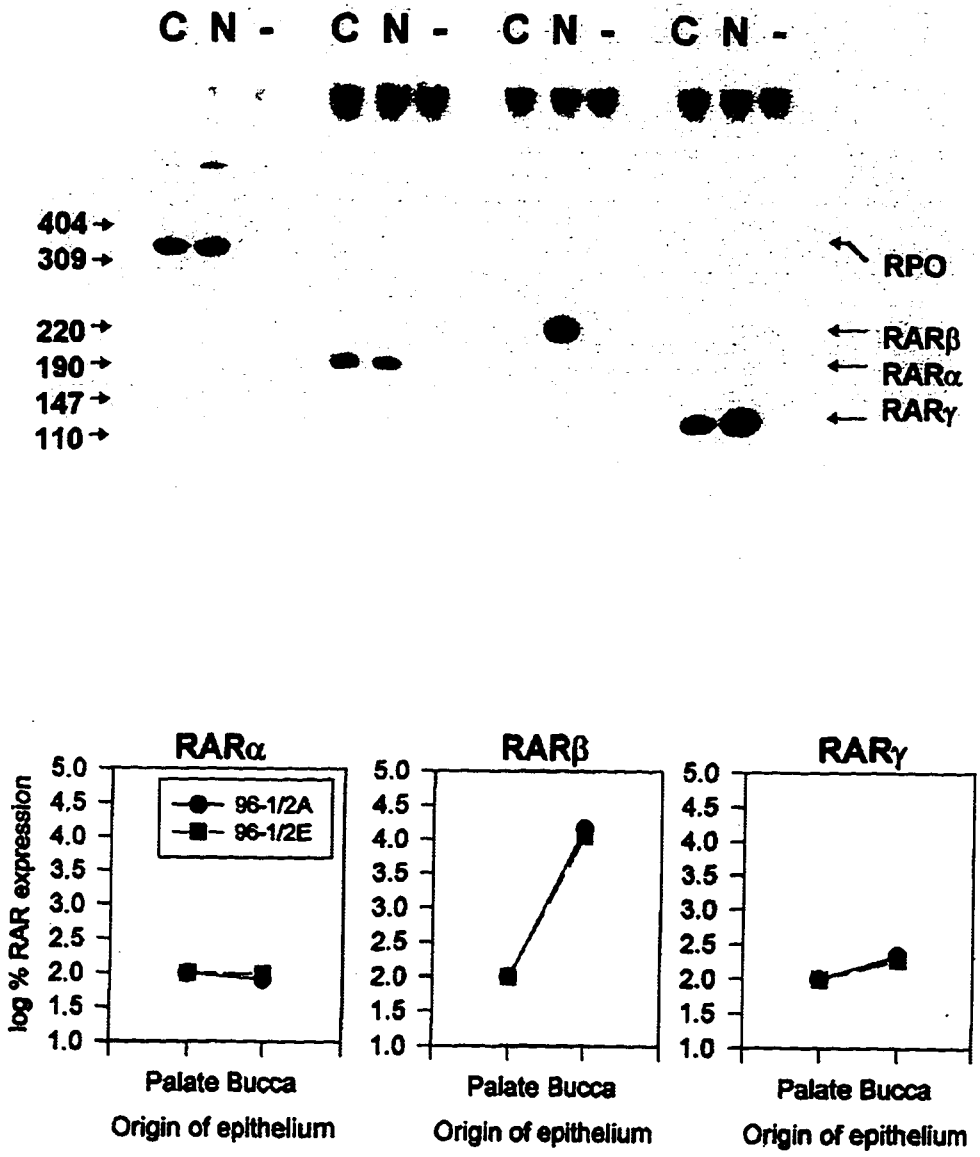

Figure 7.6: RARβ expression is many-fold higher in oral noncornified epithelium as compared to epithelium from a cornified region. The upper part shows an autoradiogram from a representative PCR quantification. Shown are amplifications of the control message, ribosomal phosphoprotein (RPO) and the three RAR subtypes (RARα, RARβ and RARγ). Origin of the two epithelia is indicated above each lane as C (cornified epithelium from the palate) and N (noncornified epithelium from the buccal fold). The third lane in each panel is a negative control amplification for each product. Marker sizes in base pairs are indicated on the left. The lower part shows a graphic representation of the RAR quantification with an additional PCR experiment included. Each RAR subtype is plotted as log% expression, where the expression in the cornified epithelium has been set to 100%. Data shown are from two separate RT reactions (see Table 7.5).

6. Summary and conclusions:

In this part of the study, it was shown that oral keratinocyte RARβ expression was increased in the order of 100-fold as epithelial differentiation was switched from a cornified pattern to a noncornified type in the organotypic cultures. In contrast, levels of RARα and RARγ messages varied only slightly in these conditions. RAR expression was determined by semiquantitative RT/PCR experiments. Although, this method was prone to technical difficulties, the above results were convincingly shown in two separate culture experiments, with each quantification being at least duplicated using cDNA templates from two separate RT reactions. In addition, two preliminary experiments showed results with the same trends in regard to RARβ expression. Finally, the results of RAR expression from cultured keratinocytes were substantiated by quantification of the receptor messages from two oral epithelial tissues. In a noncornified oral epithelium covering the buccal mucosa, RARβ expression was many fold higher than its expression in a cornified oral epithelium which covers the palate. RARα and RARγ message levels were similar in the two epithelia. Interestingly, the relative difference in RARβ expression between the two patterns of epithelial differentiation was comparable in the culture conditions and *in situ*. Thus, these results convincingly showed an inverse relation between RARβ expression and oral epithelial cornification.

CHAPTER VIII

Effects of RAR subtype-specific ligands on oral epithelia grown in organotypic raft cultures

1. Introduction:

In an effort to better understand the observed association between the degree of oral epithelial cornification and RARβ expression, experiments with RAR subtype-specific ligands were undertaken. The aim of this part of the project was to obtain evidence for a cause and effect relationship between RARβ and regulation of oral epithelial cornification by use of two synthetic retinoids which preferentially bind and activate specific RAR subtypes. Thus, on the basis of the association between RARβ expression and oral epithelial cornification (see chapter VII), it was predicted that a RARβ selective agonist would induce the cultured epithelia to undergo a similar switch in cornification as was evident in the previous experiments with RA. As a negative control, a RARα selective agonist would be used with the expectation that this ligand would not bring about the RA induced changes in epithelial differentiation. Such results would strongly support the hypothesis that RARβ is instrumental in the differentiation switch between cornified and noncornified epithelia (see Chapter VII).

2. Background:

The use of specific ligands at the receptor subtype level is one way to investigate the specific function(s) of the RARs (see Chapter I). Since naturally occurring ligands with such specificities are not yet known, synthetically produced compounds have been employed in such studies. Experiments with synthetic retinoids in biological systems were first performed more than twenty years ago (Bollag, 1975), but reports of RAR

subtype-specific retinoids appeared only recently after the discovery of the RARs (Kagechika et al, 1988; Delescluse et al, 1991). Much of the current progress in this field is driven by the desire to produce synthetic retinoids with very specific therapeutic actions, thus avoiding the wide range of serious side effects that accompanies current pharmacological retinoid protocols (Gollnick, 1996).

The basis for the RAR subtype-selectivity towards certain synthetic retinoids can be found in the E-domain, the ligand binding domain, of the RARs (see Chapter I). Although a high degree of homology exists in this part of the molecule between the three RARs, the homology does not reach 100%. A comparison of the three RAR subtypes reveals that three key amino acid residues vary within the ligand binding pocket of these proteins (Renaud et al, 1995). Because all-*trans* RA contains alternating double and single bonds in its polyene chain (Figure 8.1), it is a relatively flexible molecule which can change its conformation and so interact with all three RAR subtypes. In the development of the selective retinoids, the conformational mobility of the molecule has been restricted by replacement of the polyene chain with ring structures and/or triple bonds (Chandraratna, 1996). The increased rigidity of thus modified retinoid molecules is responsible for the selective fit into a specific RAR ligand pocket. An example of early selective retinoids is the Am 580 molecule (Figure 8.1) which has an approximately 30-fold higher binding affinity for RARα than RARβ and RARγ (Kagechika et al, 1988). Further modifications of this molecule led to the synthesis of AGN 193835 (Teng et al, 1996) which has an even higher selectivity for RARα (Table 8.1) and was, therefore, used as the RARα selective agonist in the present study. Another set of modifications led to the synthesis of the RARβ selective agonist, AGN 193174[1] (Johnson et al, 1996), which was the RARβ selective retinoid used in the present experiments (Figure 8.1).

[1] AGN 193835 and AGN 193174 were generously provided by Dr. R. Chandraratna, Allergan, Irvine, CA.

Table 8.1: Binding affinity K_d (nM) of selected retinoids to RARs alpha, beta and gamma.[a]

	RAR		
retinoid	α	β	γ
RA	15	13	18
AM 580	36	1361	3824
AGN 193835[b]	4.4	3037	>30000
AGN 193174[c]	129	20	104

[a] Adapted from Teng et al, 1996 and Johnson et al, 1996.
[b] Used as the RARα selective agonist in the present study.
[c] Used as the RARβ selective agonist in the present study.

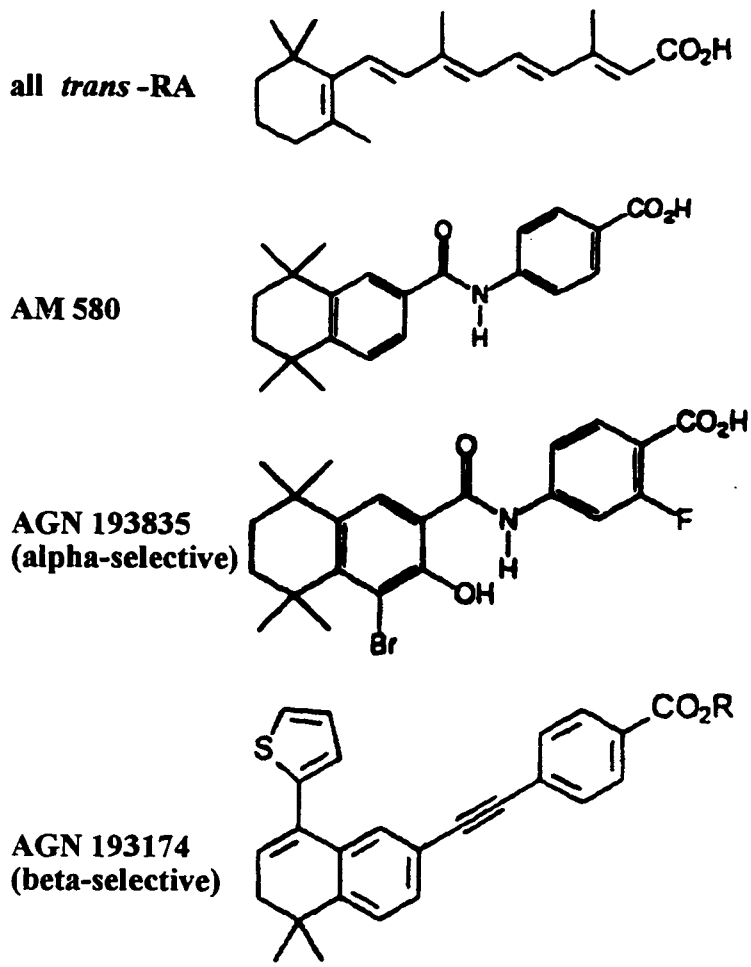

Figure 8.1: Structures of all *trans*-RA, AM 580, AGN 193835 and AGN 193174. Adapted from Teng et al, 1996 and Johnson et al, 1996.

3. Methods:

The experiments with these synthetic retinoids were performed using the organotypic culture model essentially as described previously (Chapter II), except for a few modifications. In an effort to minimize the inter-experimental variation between experiments, two keratinocyte strains (HOKs 96-12 and HOKs 96-13) were produced. Both these strains were derived from gingival tissues as described in Chapter II, with the exception that these keratinocytes were expanded in primary monolayer cultures only. The secondary cells were then frozen overnight at -80°C in aliquots of approximately $1x10^6$cells/mL freezing medium[2] and stored in liquid nitrogen. For the experiments at the air-liquid interface, the keratinocytes were thawed and expanded on plastic in defined medium (KBM, Clonetics), then passaged and seeded on the collagen lattices as tertiary cells. A second major change was in the use of commercially available delipidized serum (Sigma) in the growth medium during the last two weeks of the culture at the air-liquid interface. This serum originated from bovine calves and was delipidized by charcoal stripping.

One experiment with each keratinocyte cell line was performed. Strain HOKs 96-13 did not grow well post-freezing and did not provide interpretable data in the organotypic culture (data not shown). The experiment using strain HOKs 96-12 included cultures with increasing RA concentrations in the medium (0mol/L, 10^{-9}mol/L and 10^{-7}mol/L) as a positive control for the retinoid effect. The two synthetic ligands, selective for RARα (AGN 193835) and for RARβ (AGN 193174) were both used at 10^{-7}mol/L and at 10^{-6}mol/L. These concentrations were somewhat arbitrarily chosen, because of the relatively unknown effects of these ligands in biological systems. The

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[2] Recipe for freezing medium:
7.2mL DMEM (Dulbecco's Modified Eagle Medium, low glucose, Gibco, Grand Island, NY)
2mL FBS (Fetal Bovine Serum, Hyclone, Logan, UT)
.834mL DMSO (dimethyl sulfoxide, Sigma Chemical Company, St. Louis, MO)

10^{-7}mol/L concentration was to be directly compared with the sample grown in the same RA concentration and the 10^{-6}mol/L concentration was chosen in case the biological efficacy of the synthetic ligands was less than that of RA.

4. Results:

Increasing RA concentrations in the growth medium were inhibitory for the degree of cornification of the cultured epithelia (Figure 8.2). In absence of RA, the epithelium exhibited paracornified morphology with a distinct granular cell layer immediately beneath the cornified cell layers. At 10^{-9}mol/L RA, the epithelium remained paracornified, but there were no signs of granularity in the transition between the differentiating cell layers and the cornified cell layers. Finally, the epithelium grown in 10^{-7}mol/L did not show any tendencies to cornification. This decrease in epithelial cornification in response to RA was also seen in the expression of proFG/FG (Figure 8.2). ProFG was expressed in a distinct granular cell layer in the RA-free condition, but its expression was absent in the higher RA concentrations. These results largely reproduced earlier experiments and confirmed that the modifications to the model system as described above had no deleterious consequences on the RA responsiveness of the keratinocytes.

Addition of 10^{-7}mol/L AGN 193835 (RARα agonist) to the medium resulted in an epithelium that largely resembled the epithelium grown in the same concentration of RA. It showed a morphology without signs of cornification (Figure 8.3), although the cells in the uppermost layers were somewhat more flattened. When the concentration of AGN 193835 was increased to 10^{-6}mol/L, the epithelium became disorganized with large vacuoles. This morphological pattern was previously seen in several experiments using a high RA concentration in the medium (for example, see Figure 6.3) and is believed to be a result of retinoid toxicity. From these results it appeared that the RARα agonist mimicked the effect of RA on oral epithelial differentiation.

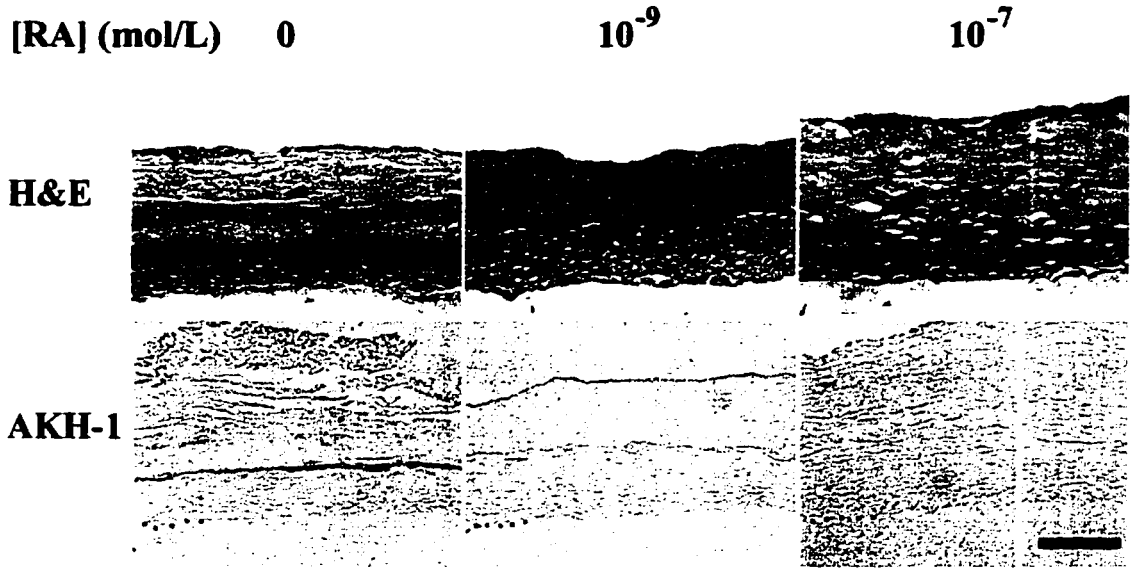

Figure 8.2: RA inhibits cornification of oral keratinocytes in organotypic culture. Oral keratinocytes were grown in organotypic culture as described (see Chapter II and Chapter VIII above) in media with increasing RA concentrations (0, 10^{-9}, and 10^{-7}mol/L RA). The cultured epithelia were assessed for morphology by Hematoxylin and Eosin staining (H&E) and for expression of proFG/FG by probing with the AKH-1 antibody. Dotted lines mark basal epithelial border and bar is 100μm. Vertical line is a scanning artifact.

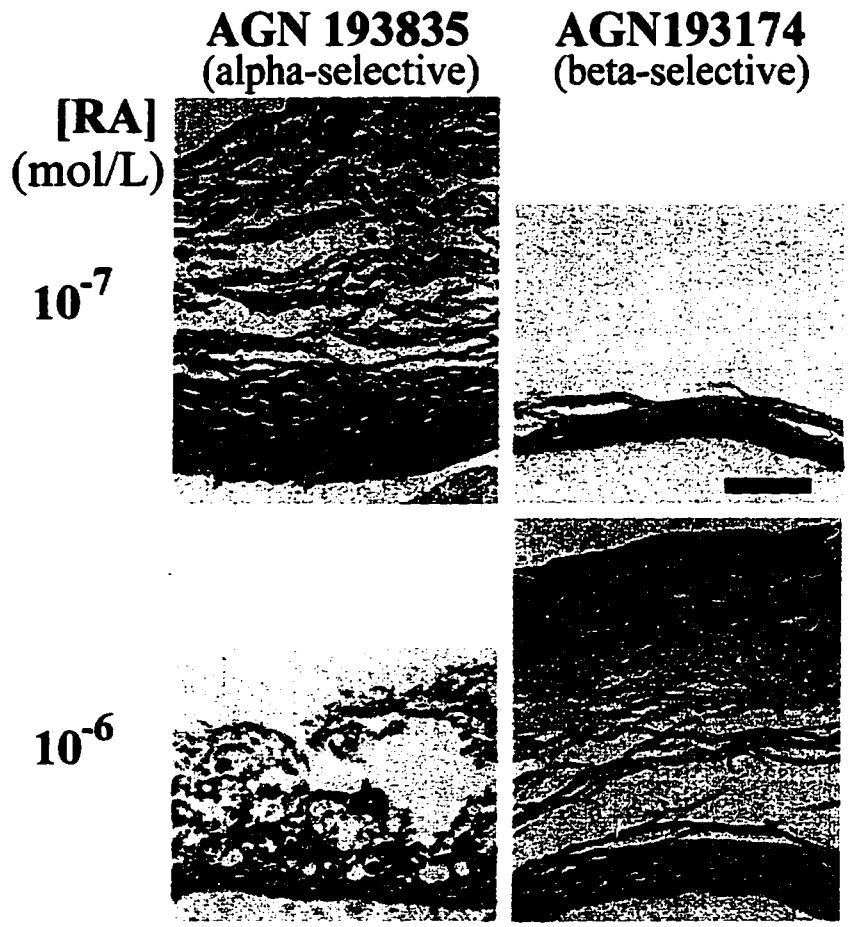

Figure 8.3: The RARα selective ligand (AGN 193835) largely recapitulates the inhibitory effects of RA on oral keratinocyte cornification, while the RARβ selective ligand (AGN 193174) does not. Parallel cultures to those shown in Figure 8.2 were grown with 10^{-7} or 10^{-6}mol/L of either AGN 193835 or AGN 193174. The epithelia were fixed, sectioned and stained with Hematoxylin and Eosin for assessment of morphology. Bar is 100μm.

In contrast, when AGN 193174 (RARβ agonist) was added to a concentration of 10^{-7}mol/L to the medium, the epithelium remained cornified with distinct signs of granularity below the cornified cell layers (Figure 8.3). Interestingly, proFG was not expressed in this epithelium (data not shown). However, the lack of proFG expression in the paracornified epithelium grown in 10^{-9}mol/L RA (Figure 8.2) shows that proFG expression is not obligatory for this type of epithelial differentiation. The most notable feature of the epithelium grown in 10^{-7}mol/L AGN 193174 is the lack of suprabasal spinous cell layers. The basal cells seemed to immediately differentiate to granular and cornified cells. When 10^{-6}mol/L AGN 193174 was added to the culture medium, the thickness of the epithelium became somewhat normalized, but tendencies to paracornification as flattened, loosely arranged cells could still be detected in the uppermost layers.

5. Summary and conclusions:

The above observations using RAR subtype specific retinoids must be viewed as a pilot study for future experiments. The RARβ selective retinoid did not recapitulate the RA effects on epithelial differentiation seen in the organotypic culture. This result suggests that RARβ is not responsible for the switch between the cornified and the noncornifed differentiation patterns. Yet, the extreme thinning of the epithelium in the 10^{-7}mol/L conditions, implicates RARβ in some regulatory event(s) of epithelial differentiation or possibly proliferation. In contrast, the RARα selective agonist was able to change epithelial differentiation in a similar manner to the switch mediated by RA.

CHAPTER IX

Discussion

1. Summary of results:

The aim of this work was to evaluate the effect of retinoic acid (RA) on oral epithelial differentiation and the possible roles of the retinoic acid receptors (RARs) in this process by correlation of the pattern of differentiation with the expression of RAR subtypes. An air-liquid interface culture system was adapted for growth and differentiation of oral keratinocytes. This culture system was used to show that RA inhibits cornified differentiation and expression of biochemical markers of keratinization of oral epithelial cells irrespective of the original *in situ* keratinocyte differentiation. It was further shown that subepithelial fibroblasts play a modulatory role in the regulation of the apparent RA-responsiveness of oral keratinocytes and their subsequent differentiation. A major finding of this study was that RARβ mRNA expression is inversely associated with cornification of the oral epithelium and that the magnitude of changes in RARβ expression dramatically overshadow minor changes in RARα and RARγ expression. The association of RARβ and oral epithelial differentiation was first observed in cultured oral epithelia in which the degree of cornification was manipulated by varying the concentration of RA in the media alone or by varying the RA concentration in the media together with the use of different fibroblast strains in the underlying collagen matrix. Subsequently, this association was confirmed directly in oral epithelia of two diverging patterns of cornification. In the latter analysis, RARβ expression was determined without interference of the underlying connective tissue and therefore represents RARβ expression in the epithelial component of the oral mucosa. It was clearly shown that a noncornified oral epithelium (from the buccal vestibule) expresses many-fold higher levels of RARβ than a cornified epithelium (from the hard

palate), while RARα and RARγ levels are similar in the two epithelia. Finally, use of a RARα specific ligand did recapitulate the RA effects on oral epithelial differentiation in the organotypic culture system, while exposure to a RARβ specific ligand did not. These preliminary results suggest that the role of RA and RARs in epithelial differentiation is more complex than previously envisioned and that the RARβ increase is a secondary event to the switch in epithelial cornification.

2. Effects of RA on oral epithelial differentiation:

The observation that RA acts as an inhibitor of oral cornified keratinocyte differentiation parallels the findings of Asselineau et al (1989), who demonstrated a similar RA effect on epidermal differentiation. Asselineau and coworkers showed that a specific range of RA concentrations (10^{-9}-10^{-8}mol/L) was required to obtain *in vitro* epithelial architecture closest to that found *in vivo*. A lower optimal RA range was found in the present study; in fact, the oral keratinocytes seemed to differentiate equally well in delipidized media with no added RA as in delipidized media with low RA concentrations (10^{-10}mol/L). This difference in RA levels for optimal cornification between the two studies may be due to differences in culture conditions and/or to inherent differences between epidermal and oral keratinocytes. Indeed, the results shown in Chapter III highlight some growth requirement differences between foreskin epithelial cells and gingival keratinocytes. This in turn necessitated the changes made in culture conditions for this project as compared to the Asselineau study. In spite of these differences, however, the general effect of RA on keratinocyte differentiation remained.

In the environment of the oral cavity, a cornified epithelium is found in the palate and the attached gingiva. A noncornified epithelium found in the lining mucosa, is also a normal pattern of differentiation in the oral cavity and the cornified and noncornified epithelia are in close proximity to each other (see Figure 1.1). Such physical proximity make it unlikely that RA regulates oral epithelial differentiation in the same manner as in

the culture model, i.e. by abrupt variation in RA concentration in the immediate vicinity of each cell. Instead, this regulation may be brought about by differences in the RA reactivity among the different keratinocytes. It was therefore of interest to consider if keratinocytes from different regions of the oral cavity respond in a different manner to RA. In contrast to the apparent differences in RA-sensitivity between widely different keratinocyte types (e.g. oral versus epidermal keratinocytes; see above), it was shown that two oral keratinocyte types react similarly to RA. One of the two keratinocyte types used in this experiment originated from the cornified gingival epithelium, and the other was from the closely approximating, but noncornified epithelium of the lining mucosa. These two cell types formed very similar epithelia in the organotypic culture model as judged by morphology and three out of the four markers of differentiation used in this study. Only K19 expression seemed to be inherently different between the two oral keratinocyte types. It is reasonable to assume that keratinocytes that are ontogenetically further apart (e.g. epidermal versus oral) would retain phenotypic differences in the culture because of their genetic programming. On the other hand, keratinocytes that are developmentally closer (e.g. oral cornified versus oral noncornified) express a similar phenotype in the same culture conditions. This would indicate that most of the signals that specify the regional variation in terminal differentiation of oral keratinocytes come from the surrounding environment. Yet, a certain degree of genetic preprogramming (e.g. K19 expression) seems to be present in the two oral cell types as well, and most likely contributes to the final phenotype of the terminally differentiated keratinocyte.

The conclusions from the above comparison must be considered with some caution. First, there were differences in RA reactivity between keratinocytes from different individuals. In an attempt to control for this confounding factor, the above comparison was done on cells from the same individual, but this was only possible for one subject. Second, in spite of histological assessment of the tissue of origin for the keratinocytes, there was an inherent difficulty in determining the origin for the oral

tissues (see below; section 5a in this Chapter). Thus, a contamination of one cell type with the other in the above experiment could not be absolutely excluded.

In spite of these short comings, there were other indicators throughout this project which suggested that each oral keratinocyte is not rigidly predetermined to follow a narrow path of differentiation, regardless of the surrounding environment. The observations from a series of experiments using primary keratinocytes from both noncornified and cornified oral mucosa (see Table 4.1), indicated that the cultured cells formed epithelia exhibiting various degrees of cornification, not necessarily consistent with their tissue of origin. In addition, oral keratinocytes from both cornified and noncornified regions could be similarly redirected in their choice of differentiation pattern by adjustments of their RA exposure and finally, they were also similarly sensitive to the cocultured fibroblast type in this regard.

In contrast to these findings, evidence of inherent differences between various oral keratinocyte populations was presented by Lindberg and Rheinwald (1990), who identified three distinct subtypes of human oral keratinocytes based on differentiation patterns in xenografts subsequent to primary cultures. However, these authors based their conclusions of intrinsic differences between oral keratinocytes on expression of a single marker in these cells, namely K19. Interestingly, if K19 was examined as the only marker of differentiation in the present study, the same conclusion would have been reached. The results in the present study, however, indicate that regional variation in oral epithelial differentiation cannot be entirely explained by intrinsic differences in the two keratinocyte types and their intrinsically predetermined response to RA. This study clearly shows that other markers of differentiation (e.g. K1) are more susceptible to extrinsic influences than to the intrinsic preprogramming of the oral keratinocyte. The K1 susceptibility to extrinsic signals was actually also shown in the Lindberg and Rheinwald study (page 233, Table 1), but was ignored in their conclusions. Thus, it is reasonable to conclude that the regional specialization of oral keratinocytes ultimately depends on an exquisite interaction between intrinsic, genetically preprogrammed

factors, and extrinsic, environmental signals. It is not clear whether the hypothesized difference in RAR expression is intrinsically predetermined or regulated by extrinsic factors. However, the present study suggests that part of the extrinsic signaling leading to the choice of oral keratinocyte differentiation is mediated by subepithelial fibroblasts.

3. Effects of fibroblasts on oral epithelial differentiation:

Subepithelial oral fibroblasts cocultured with oral keratinocytes influenced differentiation of the epithelial cells in accordance to their tissue of origin via an apparent modulation of keratinocyte RA-sensitivity. Consequently, fibroblasts from cornified oral mucosa inhibited the RA-response of oral keratinocytes, thus leading to more abundant expression of markers of cornification in the epithelial cells, while fibroblasts from noncornified epithelia potentated the RA-response of oral keratinocytes. The magnitude of the fibroblast influences varied widely, depending on the fibroblast type examined. Differences in keratinocyte RA-sensitivity were greatest in comparisons between unrelated fibroblast types, e.g. embryonal epidermal fibroblasts (GM10) versus adult oral fibroblasts, but diminished in comparisons of closely related fibroblasts, e.g. among different strains from the oral cavity. In the latter comparison, the fibroblast effects were clearly less striking than the effects exerted by RA. These results are in agreement with many studies that show the influence of connective tissues on epithelial differentiation (see below). However, the observations made in this study suggest that the subepithelial fibroblasts are major players in these influences and that these effects are mediated by modulating the RA-response of the keratinocytes.

Although the influence on keratinocyte differentiation by fibroblasts in accordance to their tissue of origin was generally consistent, there were exceptions (e.g. experiment 94-4, Figure 7.1) As discussed above, some of the variation in these results may have been due to the difficulty of correctly assessing the differentiation pattern of the original site of the fibroblasts. The removal of the healthy tissues should ideally be

done under controlled conditions in order to avoid any uncertainty about the site origin of each cell type. This was, however, not practically possible. Furthermore, the above comparison was done using fibroblasts from different subjects and interindividual differences may have contributed to the variability. Ideally, these experiments should be done with the various recombinations of fibroblasts and keratinocytes taken from two differing oral regions of one subject. Again, practical limitations of this approach have not allowed the execution of such an experiment.

As discussed above, some previous studies suggest that the adult oral epithelial phenotype is an intrinsic property of the epithelium (Ouhayoun et al, 1988; Lindberg and Rheinwald, 1990; De Luca et al, 1990), while other observations indicate that connective tissues are responsible for the final pattern of oral epithelial differentiation (Karring et al, 1975; Mackenzie and Hill, 1984; Boisnic et al, 1993). The present study suggests that neither the intrinsically predetermined keratinocyte phenotype (see Figure 4.5), nor the influence of regional fibroblasts (see Figure 6.7) are by themselves capable of inducing the large variation in terminal differentiation seen in the oral cavity. This suggests that there may be a continuous cross talk between the oral keratinocytes and the oral fibroblasts during the induction process leading to a modulation of each signal and consequently leading to a distinct choice in a specific terminal differentiation pathway. In addition, other factors, not considered in this study, may contribute to the final choice of differentiation taken by the keratinocytes. These factors may consist of signals from other circulating hormones, such as T3 (discussed in Chapter V), signals generated via direct contact of adjacent extracellular matrix (Watt et al, 1993) and possibly even neural signals. How each signal is integrated in the cellular machinery of the keratinocyte to exert its effects on gene transcription and subsequent choice of differentiation is at present largely unknown.

In the present study, it was shown that subepithelial fibroblasts influenced the apparent RA-sensitivity and hence the differentiation of oral keratinocytes. This could theoretically be achieved by two fundamentally different mechanisms:

One mechanism may simply depend on various degrees of "filtering" of retinoids by the fibroblasts. In this scenario, fibroblasts from a cornified epithelium would retain or otherwise inactivate more of the retinoids as they diffuse from the circulation (or culture medium) to the epithelium. This would have the consequence that less retinoids would be made available to the keratinocytes of the cornified as compared to the noncornified epithelia with the subsequent RA-regulatory effects on choice of differentiation as described above. Practical considerations make such a mechanism rather unlikely. Neither *in situ* nor in the cultured collagen lattices is the subepithelial fibroblast population dense enough to form an effective barrier and thus a control gate for the retinoids. In addition, it has sofar not been reported that noncornified epithelia contain a higher concentration of retinoids than cornified epithelia.

The second mechanism may be mediated by tissue specific expression of one or more soluble fibroblast products that influence epithelial cells. Indeed, Boukamp et al (1990) and Mackenzie et al (1993) have demonstrated that dermis regulates epidermal keratinocyte growth and differentiation via diffusable factors. This diffusable signal could potentially intersect the retinoid signaling pathway at one of several levels; at the level of one of the enzymes responsible for conversion of retinol to RA (Fisher and Voorhees, 1996), at the level of the CRABPs through their proposed ability to sequester RA in the cytoplasm of epithelial cells (Astrom et al, 1991) and finally, at the level of the RARs and a direct effect on transcription of genes. Neither the identity of the soluble factor(s), nor its potential mechanism to affect the RA signals are currently known. In fact, it is not even known whether the fibroblast influences are mediated via the RA-dependent signaling pathways.

4. RAR expression in oral epithelia and associations with epithelial cornification:

RARβ expression was inversely correlated with degree of oral epithelial cornification. This association was found both in the organotypic cultures and in an *in situ* comparison between cornified and noncornified oral epithelia and the magnitude of the RARβ increase was similar in both systems. In contrast, RARα and RARγ expression remained practically unchanged in the different patterns of epithelial cornification. Although RARβ expression is known to be upregulated in response to RA (de The et al, 1989), the *in situ* determination of RARβ levels showed that the association between expression of this receptor and oral epithelial differentiation is not simply due to the "artificial" RA exposure used in the organotypic cultures. The results of this study confirm and extend the observations of Crowe et al (1991), who found a relation between RARβ and K19 expression in eight of ten cultured oral keratinocyte strains. Based on this observation and on the fact that K19 expression is associated with a noncornified keratinocyte phenotype (Bartek et al, 1986; Kopan et al, 1987), they suggested that RARβ plays a role in regulation of squamous epithelial differentiation. The major findings of the present study offer support for this contention both from *in vitro* (cultures) and *in situ* (tissues) experiments and extend the relationship to morphologic patterns of differentiation and expression of additional markers. Further support consistent with this role of RARβ comes from experiments in mouse cervical epithelia (Darwiche et al, 1994), where of the three RAR subtypes only RARβ was downregulated in association with squamous metaplastic foci. The above studies are three independent findings of an inverse association between RARβ expression and cornification or indirect signs of cornification of mucosal (nonepidermal) epithelia. This association is strengthened by the fact that the three studies used widely different model systems and techniques. So, for example, Crowe and coworkers used Northern blots to detect and quantify RARβ expression, Darwiche and coworkers used *in situ* hybridization for this purpose and in the present study, the more sensitive RT-PCR

technique was utilized. Of these three techniques, only the Northern blotting is appropriate for analyses aiming at absolute quantification of the messages. Crowe's group found a maximal four-fold increase of RARβ expression in oral keratinocytes upon exposure to RA. However, these findings were done in experiments using conventional, submerged culture techniques which do not allow proper differentiation of the keratinocytes and therefore, extrapolation to *in vivo* conditions must be done with caution. In the present study, RARβ was found to increase between 25- and 300-fold in the culture experiments and there was an approximately 100-fold difference in RARβ expression in the *in situ* comparison between oral cornified and noncornified keratinocytes. However, because of the semi-quantitative nature of the RT-PCR technique used in these analyses, the absolute value of the increase remains uncertain. Nevertheless, the inverse relation between RARβ expression and nonepidermal cornification is clearly shown in the present study and is further supported by similar findings in the two studies quoted above. In addition, the three studies also agree in their findings that RARα and RARγ remain relatively unchanged in the various differentiation patterns of mucosal epithelia.

5. Shortcomings of the experimental approach in the current study:

5a. Assessment of RA effects on epithelial differentiation:

As shown in Table 4.1 there was a considerable variation in the RA-sensitivity of the oral keratinocytes in consecutive experiments. This variation was seen in spite of efforts to rigorously standardize the culture technique both during the expansion phase of the cultures and during the cell growth in the organotypic phase. Therefore, this variation can most likely be attributed to the use of separate tissue donors for each experiment. With this approach, factors like the donors' gender, age, oral hygiene and donor site may have contributed to the differences seen between each experiment. However, the variation in RA-sensitivity of the oral keratinocytes observed between

consecutive experiments does not alter the interpretation and conclusions of the results reported. The general effects of RA on oral keratinocyte differentiation and the influences of the various fibroblasts were consistently observed. Furthermore, to insure reliability, most of the results shown in this report were derived from comparisons within single experiments which were repeated several times, and any direct comparisons between experiments were made with caution.

Attempts to adjust for (or at least to understand) the effects of donor site were pursued by examining a representative piece of each donor tissue by means of morphology and expression of markers of differentiation. In this manner, each tissue biopsy was classified into one of three groups in respect to its original differentiation pattern; cornified, noncornified or transitional (containing areas of both cornified and noncornified epithelia). As shown in Table 4.1 and in Figure 4.5 and by other experiments not shown or tabulated, there did not seem to be a relation between the site of origin of the tissue and the RA-sensitivity of the keratinocytes. This conclusion, however, has a few caveats: First, although it was always attempted to take a representative piece of each tissue for the determination of site of origin, it is impossible to known whether the chosen piece was truly representative for the rest of that particular tissue. The choice of the piece for this assessment was done upon macroscopic inspection of the tissue - a technique with obvious limitations in sensitivity and specificity. Second, the morphologic and immunohistologic assessment of the epithelial differentiation turned out not to be so straightforward itself. During a part of this project, an attempt was made to accumulate enough oral tissue of cornified and noncornified origin for analysis of *in situ* RAR expression in the two epithelial types. In conjunction with this effort, a whole series of morphologic and immunohistochemical assessments of these tissues was done. In this process, it became clear that the approach of getting tissues from the oral surgeon for comparisons between the two epithelial differentiation patterns was not ideal. Two problems were identified: First, there are many regions in the oral cavity that are transitional in nature, i.e. they do not

show clear criteria for either the cornified or the noncornified differentiation pattern. Figure 9.1 shows an example of such an epithelium. Morphologically, it approximates a paracornified epithelium, but there are no visible squames in the uppermost cell layers. By markers of differentiation, it is also difficult to classify. This epithelium expresses neither K19, nor proFG/FG, but expresses both K13 and K1. Second, some tissues contain both differentiation patterns and this is not always macroscopically obvious. An example of this problem is shown in Figure 9.2. This tissue piece is covered by an epithelium showing three patterns of cornification; noncornified, paracornified and orthocornified. In this tissue, the markers of differentiation correspond well to the morphology of the epithelium. The noncornified part expresses K19 in the basal cell layer and K13 suprabasally, the orthocornified part expresses proFG/FG in the granular cell layer (although weakly) and K1 suprabasally and the paracornified part expresses a mixture of these markers. Ideally, to avoid this confusion, the tissues necessary for the experiments in the present study should have been removed under controlled conditions in order to obtain a homogeneous piece of tissue displaying either one or the other epithelial differentiation pattern. However, as discussed above, this was not practically possible and was done only once during this project, namely for the assessment of *in situ* RAR expression.

In summary, because of the above limitations in the experimental protocol, the conclusion that the overall RA-sensitivity of oral keratinocytes is independent of the site of origin of the epithelium must be viewed with some caution.

5b. Correlation of RAR expression and epithelial differentiation:

The RT-PCR technique as used in this project for determination of RAR expression is semi-quantitative in nature. In addition, the assessment of the degree of epithelial cornification is based on morphology and marker expression, and since these experimental outcomes cannot be measured precisely, this assessment is only semi-

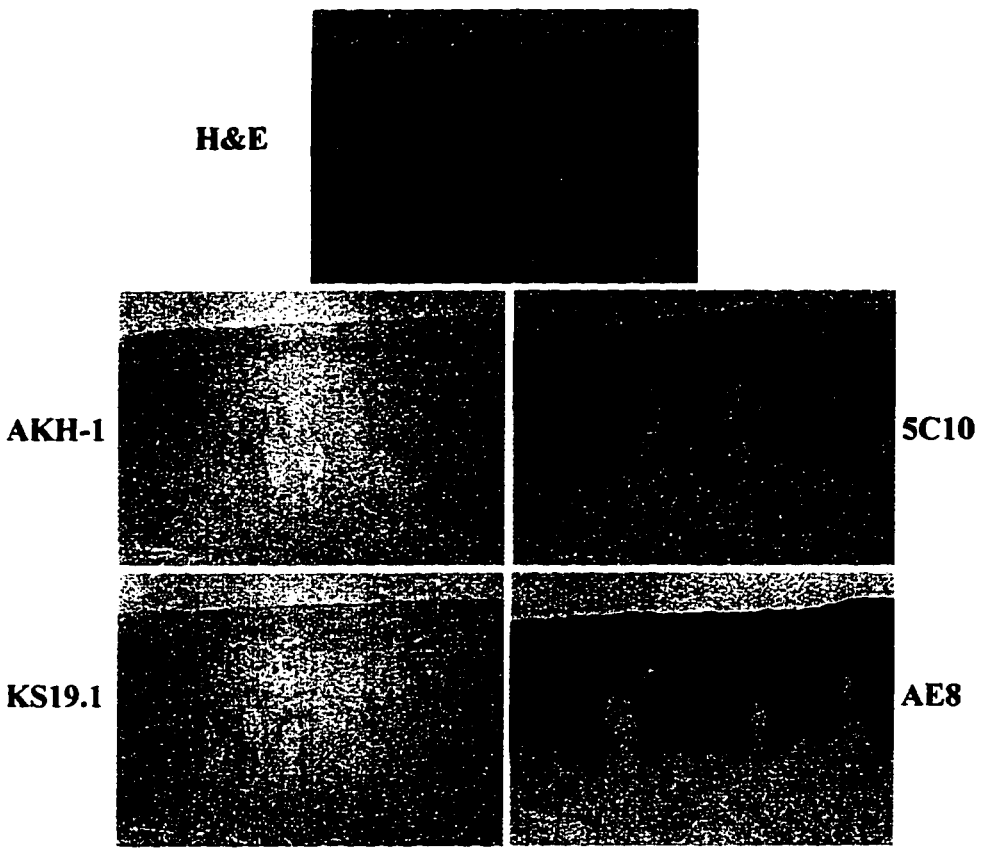

Figure 9.1: Oral epithelia can express both markers of cornified and noncornified differentiation simultaneously. Thin sections from an oral biopsy were assessed for epithelial differentiation by morphology (H&E) and by expression of markers of differentiation using immunohistochemistry. Note simultaneous absence of expression of proFG/FG (antibody AKH-1) and K19 (antibody KS19.1) and simultaneous expression of K1 (antibody 5C10) and K13 (antibody AE8). Bar is 100μm.

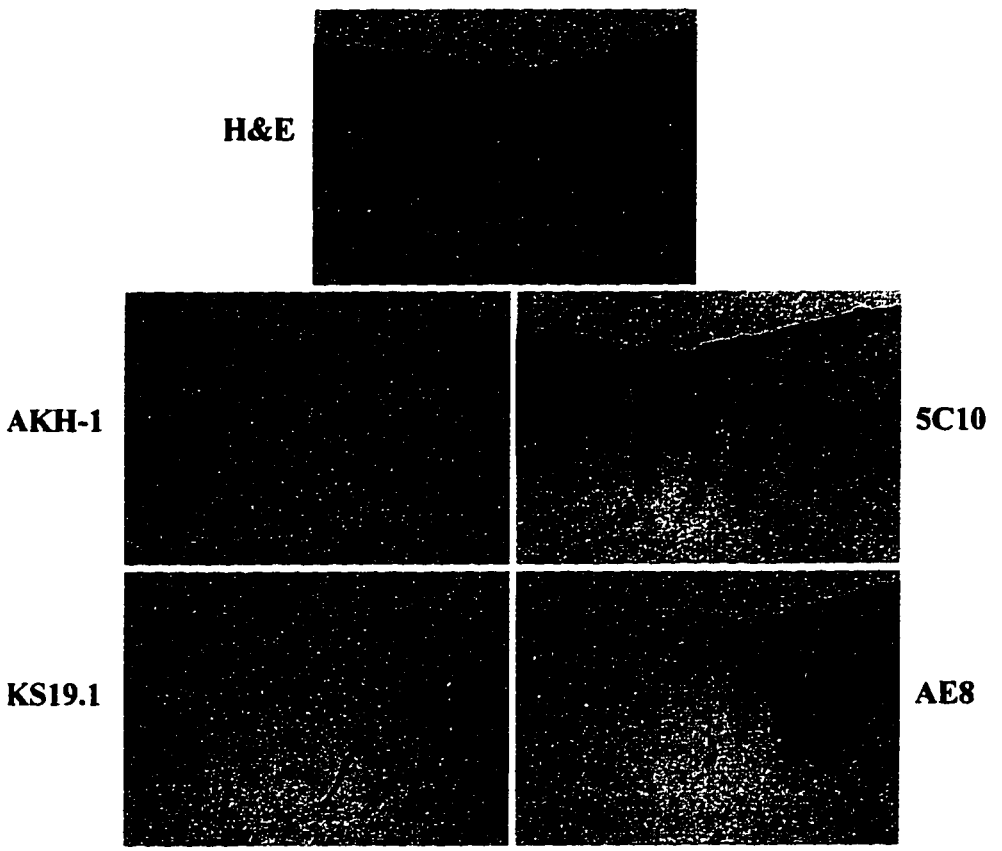

Figure 9.2: Oral biopsies can contain both cornified and noncornified epithelia. Thin sections from an oral biopsy were assessed for epithelial differentiation by morphology (H&E) and by expression of markers of differentiation using immunohistochemistry. Note a cornified differentiation pattern on the left half of this biopsy shown by expression of proFG/FG (antibody AKH-1) and K1 (antibody 5C10) and a noncornified differentiation pattern on the right half shown by expression of K19 (antibody KS19.1) and K13 (antibody AE8). Bar is 100μm.

quantitative in nature as well. The lack of absolute quantification of the two outcomes makes interexperimental comparisons impossible. However, the results reported in this thesis are based on several separate experiments, in which the association between RARβ expression and oral epithelial differentiation was reproduced.

The range and apparent variation of the RARβ increase in the results of the present study may seem large. This phenomenon cannot simply be explained by interexperimental differences, since the variation was even seen within single experiments (see Figure 7.5). One possible explanation for these large deviations may be that the RT-PCR methodology is very technique sensitive and small errors (for example in pipetting) can be amplified in the final results. Nevertheless, although exact values vary, it is important to note that the data are consistent for the relationships between each of the three RAR subtypes and keratinocyte differentiation in the multiple culture experiments presented in this thesis. Also, the relationship between RAR expression and oral epithelial differentiation was further strengthened by the observations made directly in oral epithelia. Although, these observations were limited to one set of tissues, because of the inherent difficulty to get tissues of predetermined and homogeneous differentiation patterns from a single subject, taken together, the culture experiments and the epithelial tissue analysis build a strong case for the inverse association of keratinocyte RARβ mRNA expression and oral epithelial cornification. Although there was a weak trend towards a RARγ increase with decreasing cornification, this increase was below the limits of the semi-quantitative RT/PCR technique to detect changes in mRNA expression (Singer-Sam et al, 1990) and must therefore be viewed as falling within the range of experimental variation.

6. Possible mechanisms of RARβ function in oral epithelial differentiation:

The association between RARβ expression and specific patterns of oral epithelial differentiation suggests a regulatory role of this receptor in the epithelial differentiation

process. This role is further supported by observations from pathologic cells and tissues. Hu et al (1991) were not able to detect any RARβ expression in seven of nine squamous cell carcinoma lines in spite of the fact that these cells originated in the oral cavity, where the normal keratinocytes express RARβ transcripts to various degrees. Xu et al (1994) examined RAR and RXR expression in squamous cell carcinomas and adjacent normal tissues and found a significant difference in expression between the two sites only for RARβ. In both these studies, expression of the RARβ subtype was decreased or absent in the carcinoma cells in which the ability for proper differentiation has been lost. Similarly, RARβ is expressed in normal mammary keratinocytes, but its expression is lost in immortalized normal and malignant cell lines (Li et al, 1995).

Most likely, RARβ is only one of several important regulators of epithelial differentiation. That RARβ alone is not responsible for directing the keratinocyte differentiation process is indicated by expression studies in epidermis. Elder et al (1991) and Redfern and Todd (1992) reported that RARγ is the major RAR subtype expressed in cultured human epidermal keratinocytes derived from normal, nonpathologic and well differentiated tissues, while RARβ is expressed poorly or not at all. These observations support the hypothesis that in normal keratinocytes an absence of RARβ expression promotes the cornified pattern of epithelial differentiation. In contrast, the experiments with pathologic cells suggest that in the malignant cell the loss of RARβ contributes both to dedifferentiation and loss of growth control. The implication is that the presumed function of RARβ in differentiation is heavily dependent on its context. Studies with transgenic mice also indicate that RARβ may be only one of several regulatory molecules involved in the process of epithelial differentiation. RARβ null mutant mice (involving all RARβ isoforms) generally develop and grow without gross abnormalities (Luo et al, 1995). This suggests that RARβ is not essential in its presumed regulatory function, but that other compensatory mechanisms exists, such as redundancy among the members of the RAR family (Kastner et al, 1995, review).

What are the possible molecular mechanisms for the proposed RA-dependent regulation of epithelial differentiation? This regulation can fundamentally occur by two major mechanisms: First, it can simply be executed as in the *in vitro* cultures of the present study, by regulation of the extracellular retinoid concentration. However, since the cornified and the noncornified epithelia are in such close physical proximity in the oral cavity, it is unlikely that such steep concentration gradients of the retinoids could be established and maintained in the tissues. In this regard, it would be helpful to carry out extracellular retinoid measurements of the subepithelial and epithelial tissues taken from the two divergent patterns of oral cornification. Second, the RA regulation could be established by a divergence in RA-sensitivity of the epithelial cells. The RA-sensitivity of the keratinocyte could theoretically be regulated at several levels along the pathway between the entry of the retinoid as retinol in the cell and the final effector molecules of the retinoid signal, the RARs.

As retinol enters the cell, it is bound by the cellular retinol binding protein (CRBP) (Bashor and Chytil, 1975; Fisher et al, 1995). CRBP-bound retinol functions as a substrate for one of two enzymes; lecithin:retinol acyltransferase which esterifies retinol to retinyl esters which in turn serve as stores of retinoids in the cell, and retinol dehydrogenase which initiates the process of RA synthesis. The choice between synthesis or storage occurs through a direct competition of these two enzymes (Fisher and Voorhees, 1996) and therefore, control of one or both of these enzymes could constitute the first regulatory level of RA-sensitivity in the keratinocyte. Indeed, regulation of the retinol esterifying activity has been shown in epidermal keratinocytes (Kurlandsky et al, 1996), where it is four-fold greater in basal epidermal cells than in cells of the upper layers.

In epithelial cells, the synthesized RA is bound by cellular retinoic acid binding protein-II (CRABP-II) and in this form can either be hydroxylated by all-*trans*-RA 4-hydroxylase to a virtually inactive form or be transferred to the nucleus where it interacts with the RARs. The role of CRABP-II in these processes is still somewhat obscure, but

its level of expression seems to be associated with degree of keratinocyte differentiation in an organotypic culture model (Sanquer et al, 1993) and *in vivo* (Eller et al, 1994). In addition, oral mucosa expresses approximately six times as much CRABP-II as normal skin when related to expression of CRABP-I (Siegenthaler et al, 1987; Siegenthaler et al, 1992). The enzyme, RA 4-hydroxylase, has been shown to be induced in its activity by topical retinoid treatment of skin (Duell et al, 1996). Thus, these two proteins provide further opportunities for regulation of RA-sensitivity of the cell.

The final level of this regulation might be achieved by a control over expression of the RARs. The idea that the RA-sensitivity is affected at this level is attractive, because by controlling a differential expression of the three RAR subtypes and their many isoforms, the RA-sensitivity and hence, the RA-response could be truly fine tuned.

Differentiation of oral epithelia is not just a simple choice between cornification and noncornification, but is a seemingly complicated process able to produce a terminal differentiation pattern almost anywhere on the continuum between the two contrasting epithelia. It is, therefore, likely that the RA-sensitive regulation of the differentiation process involves checkpoints at several levels along the RA-signaling pathway. Some of these may be intrinsically predetermined for a particular cell type, while others are influenced by external factors and together they affect the transcriptional machinery of the keratinocyte to form a specific phenotype of the cell. The main results of this study are consistent with the notion that RARβ is an important player in this regulatory process.

The strong association of RARβ expression and the noncornified differentiation pathway of oral epithelia does, however, not exclude the possibility that induction of RARβ expression is a secondary event as a result of the switch to the noncornified differentiation pathway. Thus, it may be involved in events other than direct regulation of differentiation in these epithelia. This notion has been recently supported by Schön and Rheinwald (1996) who showed that overexpression of RARβ in oral and epidermal keratinocytes has little effect on the differentiation process in a lifted culture model.

Unfortunately, the lack of response to RA of the control lifted cultures in these studies, cloud the authors' conclusions. Thus, this issue has not been resolved and needs to be tested in a more RA responsive model system. Nevertheless, the alternative hypothesis that the RARβ increase is a secondary phenomenon to the differentiation switch must still be entertained.

In the final part of this work, evidence was sought to examine the question whether RARβ is a primary effector of noncornified differentiation or whether it is responsive to this differentiation pattern. The pilot study described in Chapter VIII showed that a RARα selective ligand (AGN 193835) closely recapitulated the RA effects on oral epithelial differentiation, while a RARβ selective ligand (AGN 193174) failed to do so. These results suggest that RARβ is not a primary regulator of the differentiation process and instead suggest a possible role of the β-receptor in the process of epithelial turnover, cell death and desquamation. In cornified epithelia, cell death and desquamation are temporally associated with events involving formation of the cornified envelope, release of profilaggrin from the keratohyalin granules and release of lipids which form lammellar structures between the flattened cells. Therefore, these events may be directly related to cell death and desquamation of the cornified cells (Dale et al, 1997). Some of the events immediately preceding the formation of stratum corneum in the cornified epithelia are altered or absent in noncornified epithelia (Reibel et al, 1989; Squier et al, 1991), in retinoid treated keratinocytes (Schmidt et al, 1985) and in epidermal cells of transgenic mice overexpressing a dominant-negative RAR in the suprabasal cells (Imakado et al, 1995). The mode of regulation of cell death in noncornified epithelia is unknown and perhaps is mediated by RARβ. Recent evidence supporting this hypothesis has been presented by Seewaldt et al (1995) who directly showed that RARβ mediates growth arrest and apoptosis in certain breast cancer cell lines. In addition, the experiment in this study employing the RARβ specific ligand, AGN 193174, suggests that specific activation of the β receptor leads to a premature terminal differentiation of the keratinocytes.

7. Significance and conclusions:

Numerous studies have shown that retinoids inhibit carcinogenesis (Lotan 1996; review). However, the efficacy of retinoids in cancer chemoprevention studies has shown a large variation, depending on the specific retinoid used, the species and organs examined and the model of carcinogenesis employed. This confusion stems from the fact that very little is currently known about the mechanism of the carcinopreventative effects of these compounds. An indication that retinoids may mediate these effects via their control of transcription of certain genes is the finding that expression patterns of the RARs are associated with certain types of cancers (Xu et al, 1994; Darwiche et al, 1995). Therefore, understanding the role of the RARs in normal cell differentiation may greatly enhance our understanding of their functions in pathology and may lead to formulation of testable hypotheses to elucidate the mechanisms of retinoids in cancer chemoprevention.

Since vitamin A is involved so extensively in a large number of biological processes, a significant problem with the use of retinoids as pharmaceutical agents is their high incidence of side effects (Costa et al, 1995). Understanding the molecular mechanisms of these compounds in normal and in pathologic cell differentiation may further lead to development of drugs with a high specificity for the condition of interest and hence, with a better overall tolerability.

In summary, the major findings of the present study were:

1. RA inhibits the cornified type of oral epithelial differentiation *in vitro*.
2. The overall, apparent RA-sensitivity of keratinocytes originating from a cornified and from a noncornified oral region is similar. However, the RA-sensitive marker of noncornified epithelia, K19, is expressed at a higher level in keratinocytes originating from noncornified oral epithelia (as compared to cornified oral epithelia) regardless of RA concentration in the culture media.

3. The apparent RA-sensitivity of oral keratinocytes is modulated by subepithelial fibroblasts.
4. Degree of cornification of oral epithelia is inversely related to expression of RARβ both *in vitro* cultures and *in situ*. Expression of RARα and RARγ shows no association with oral epithelial differentiation.
5. Preliminary experiments employing RAR subtype-specific ligands indicate that the RARβ increase is a secondary event in response to the switch of epithelial differentiation.

In conclusion, these results suggest an important function of RARβ in the regulation of oral epithelial differentiation. Although, it may not be instrumental in regulating the switch between cornified and noncornified epithelia, it may be involved in the signals leading to cell death in the oral noncornified epithelium. The organotypic culture system used for growth of oral epithelial cells in this study and the first attempts to use RAR subtype-specific ligands in this system offer a useful model for further experiments to test molecular mechanisms of RA and RAR function in epithelial differentiation and in oral carcinogenesis.

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