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Squamous Cell Differentiation Markers in Normal, Premalignant, and Malignant Epithelium: Effects of Retinoids

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Abstract Vitamin A and some of its analogs (retinoids) maintain normal differentiation of epithelial tissues by preventing aberrant squamous differentiation of cells in nonkeratinizing epithelia. They can also reverse squamous metaplasia, which develops *in vivo* during vitamin A deficiency. These effects are the result of the ability of retinoids to suppress the expression of genes associated with squamous differentiation (*e.g.*, transglutaminase type I, loricrin, involucrin, filaggrin, and keratin K1). In addition, retinoids reverse keratinizing premalignant lesions in the oral cavity, and inhibit the growth and squamous differentiation of head and neck squamous cell carcinomas (HNSCCs) *in vitro*. Nuclear retinoic acid receptors, which function as DNA-binding, *trans*-acting, transcription-modulating factors, are considered to be the proximate mediators of the effects of retinoids on gene expression and may mediate the re-regulation of aberrant differentiation and growth of premalignant and some malignant cells, thereby suppressing the development of head and neck cancer.

Key words: Head and neck SCC, nuclear retinoic acid receptors, retinoids, squamous differentiation

Retinoids are structural or functional analogs of vitamin A, or retinol. They exert profound effects on the differentiation of epithelial cells, including those lining the oral cavity and upper aerodigestive tract. The maintenance of the mucus-secreting function of these cells depends on the continuous presence of vitamin A; its absence leads to the development of squamous metaplasia, a condition that can be reversed by supplementation with vitamin A or β-all-*trans*-retinoic acid (tRA) [1,2]. Squamous metaplasia of the upper aerodigestive epithelium is considered to be a precursor of certain cancers [3]. It is thought that agents such as retinoids, which restore the normal nonkeratinizing phenotype to premalignant and malignant lesions, may also restore their responsiveness to normal growth control mechanisms and thereby suppress carcinogenesis [4].

SQUAMOUS CELL DIFFERENTIATION MARKERS

Many of the gene products expressed during squamous differentiation are involved in the formation of the crosslinked envelope [5–7]. These products include the crosslinking enzyme transglutaminase type I (TGase-I) that is associated with the cytoplasmic face of the plasma membrane and which catalyzes the formation of covalent ε-(γ-glutamyl)lysine isopeptide linkages between protein-bound glutamine residues and primary amines, such as protein-bound lysine, present in various envelope protein precursors [8]. Substrates for TGase-I include a variety of proteins such as loricrin, a 25–30 kD protein

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found in the granular and lower cornified cell layers of epidermis [7]; cornifin, a 14 kD protein which is expressed in the suprabasal layer [9]; involucrin, a 68 kD protein which is the major protein component of cornified envelopes in cultured keratinocytes [5]; keratolinin, a 16–26 kD protein; annexin I, a 36 kD protein; and sciellin, an 82 kD protein.

Another group of squamous differentiation markers are the intermediate filaments or keratins. Specifically, in the oral cavity, the cornified epithelium of the gingiva expresses K1 keratin, whereas the noncornifying stratified epithelia covering most of the oral cavity do not produce the K1 keratin but express other keratins [10, 11]. The aggregation of keratin filaments is augmented by the protein filaggrin, a 37 kD protein derived from a higher molecular weight precursor in cornifying cells. Other squamous differentiation markers are a 16–20 kD prorelaxin-like molecule [12,13], cholesterol sulfotransferase and cholesterol sulfate [14], acylceramides and lanosterol-lipid.

NORMAL AND ABERRANT SQUAMOUS CELL DIFFERENTIATION IN THE UPPER AERODIGESTIVE TRACT

The epithelium covering the hard palate, the dorsal anterior part of the tongue, the gingiva of the oral cavity, and the esophagus resembles epidermis in that it is a stratified and keratinizing cornified epithelium [10]. In contrast, the mucosa lining the remainder of the human oral cavity, including the soft palate, the ventral surface of the tongue, the floor of the mouth, the alveolar area, the lips, and the cheeks is a nonkeratinizing epithelium [10]. Likewise, the epithelium of the tracheobronchial tree is noncornified [14]. Some columnar and transitional nonkeratinizing epithelial cells (*e.g.*, lachrymal gland, trachea, bladder, and prostate) can undergo squamous metaplasia, indicating that they have the potential to differentiate along the squamous pathway. This potential is expressed *in vivo* after mechanical injury, vitamin A deficiency, or exposure to carcinogens or tumor promoters. The precursor cell type for the aberrant squamous cell may be the basal cell or the secretory cell. In rabbit tracheal epithelial cells, squamous differentiation induced *in vitro* is accompanied by increased expression of K13 keratin. In hamster trachea, squamous metaplasia induced by vitamin A deficiency is accompanied by increased expression of K5 keratin [16]. Squamous cell carcinomas (SCC), which account for over 90% of the tumors in the oral cavity, also undergo some degree of squamous differentiation. Thus, squamous cell differentiation in the upper aerodigestive tract is usually an abnormal differentiation.

The ability to form crosslinked envelopes is retained by many premalignant and malignant cells which express TGase-I and contain protein precursors for cross-linking. For example, exposure of buccal epithelial cells to the tumor promoting agent 12-*O*-tetradecanoylphorbol-13-acetate increases involucrin levels and induces the formation of cross-linked envelopes [17]. Furthermore, involucrin is expressed in premalignant lesions and SCCs [18,19]. TGase-I is detected in benign and malignant neoplasms of the oral cavity that show squamous differentiation, but is not detected in severe oral epithelial dysplasia or undifferentiated invasive SCC [20]. TGase-I is expressed in the minority of human lung SCCs grown *in vitro* or as xenografts in nude mice, whereas involucrin is expressed in all the cell lines, suggesting that control of the expression of these two markers is not tightly coordinated [20]. The expressions of TGase-I and involucrin are similarly uncoupled in lesions of severe oral epithelial dysplasia; TGase-I expression is suppressed but involucrin is present [20]. Studies with the hamster cheek pouch model of oral cancer have established that carcinogenesis is accompanied by increased expression of TGase-I during progression from normal cells through premalignant papilloma to carcinoma [21]. In the same model, expression of K1 keratin increased in hyperplastic lesions and decreased in dysplastic lesions and carcinomas.

Although the K1 keratin is lost during carcinogenesis in mouse skin, there are reports of the presence of this keratin in squamous carcinomas *in vivo* and *in vitro* [22]. Some SCCs continue to produce the same keratins as the normal precursor cells. However, neoplasms which arise in complex stratified epithelia often differ in keratin expression from the surrounding normal tissue, possibly because they represent an expanded subpopulation with a distinct keratin synthesis pattern. For example, keratins K6 and K16 are usually present in very low

amounts in normal epithelial tissues, but are expressed in hyperproliferative states and in SCC.

EFFECTS OF RETINOIDS ON SQUAMOUS CELL DIFFERENTIATION IN NORMAL, PREMALIGNANT, AND MALIGNANT CELLS

β-Carotene serves as the precursor for *in vivo* biogenesis of retinol. β-All-*trans*-Retinoic acid (tRA) is a natural metabolite of the retinol that can replace the latter in regulating epithelial differentiation as well as many other functions *in vivo*. tRA is synthesized from retinol by various tissues and cultured cells. 13-*cis*-Retinoic acid (13*c*RA) is an analog of tRA, which is also formed *in vivo* by isomerization. It is present in human serum at concentrations similar to those of tRA. 13cRA exhibits effects similar to tRA on cell growth, differentiation, and carcinogenesis but is less toxic and causes fewer side effects *in vivo* than does retinoic acid. More recently 9-*cis*-retinoic acid has been shown to be a natural isomer of retinoic acid and its presence in kidney and liver has been demonstrated.

Numerous reports have documented the ability of retinoids to suppress squamous differentiation in normal, premalignant (*e.g.*, papillomas), and malignant keratinocytes [6,14,16,23]. Our studies with human head and neck SCC (HNSCC) have demonstrated that some exhibit markers characteristic of squamous differentiation and respond to retinoids under specific culture conditions.

Retinoids have been shown to reduce spontaneous and calcium ionophore-induced crosslinked envelope formation, especially when the cells are cultured in vitamin A-deficient medium [6,8,14,18]. This effect appears to result from suppression of both TGase-I activity and the levels of envelope precursor proteins, such as involucrin [18], loricrin [7], and cornifin [9]. Human and rabbit tracheal epithelial cells cultured in a serum-free, defined medium responded to very low retinoid concentrations by suppressing TGase-I activity and inhibiting envelope formation [14]. Several studies with a variety of cultured cell lines established from SCCs of the human oral mucosa have demonstrated that different retinoids inhibit TGase-I, decrease involucrin content, and suppress the formation of cornified envelopes [18,22]. The suppression of TGase-I is presumably at the transcriptional level. The effect is two-fold in that retinoic acid not only inhibits the expression of TGase-I during the growth of tracheobronchial epithelial cells, but also reverses already squamous-differentiated cells. Retinoic acid exerts two distinct effects on loricrin expression: it not only blocks calcium-induced loricrin mRNA synthesis, but also suppresses elevated loricrin mRNA levels in differentiated cells that have been pretreated with calcium [24]. The expression of another envelope precursor, cornifin, is similarly inhibited by retinoids in rabbit tracheal epithelial cells and in human keratinocytes [9].

We studied the effects of RA on the expression of TGase-I in eight cell lines derived from human HNSCCs of the oral cavity and found that tRA suppressed the expression of this marker to different degrees [25]. In one of the cell lines, HNSCC 1483, the potential to crosslink proteins in the presence of the Ca^{2+} ionophore Ro2–2985 was suppressed by tRA [25]. A similar suppression of envelope competence by RA was reported for normal keratinocytes, a premalignant papilloma cell line, malignant SCC-13 cells from a tumor of the facial skin, and buccal SqCC/Y1 cells [22]. The tRA effect on envelope competence was the result of both a suppression of TGase-I and a decrease in involucrin expression at the mRNA and protein levels [Zou and Lotan, unpublished].

A physiological role of vitamin A in the regulation of keratin synthesis is implied by examination of changes in keratin expression following vitamin A deficiency in experimental animals. Vitamin A deficiency in rabbits is accompanied by increased keratinization of conjunctival and corneal epithelia and by corresponding changes in keratins. Whereas the normal corneal and conjunctival epithelial cells do not express significant amounts of the 56.5 kD K10 and 65–67 kD keratins K1 and K2, they do express these keratins during vitamin A deficiency [26]. Similar changes in keratins can be detected in esophageal epithelium, although this epithelium does not seem to morphologically undergo keratinization during vitamin A deficiency; it has been suggested that the biochemical changes may precede morphological keratinization. Many cell lines established in culture from SCCs suffer from a defect in terminal

differentiation, producing large amounts of the 40 kD keratin and very low amounts of the 67 kD keratin. Reducing the level of vitamin A in the growth medium by delipidation of the serum supplement induces these cells to produce more of the 67 kD keratin and less of the 40 kD keratin. The level of 67 kD keratin produced under these conditions is still lower than that produced by normal keratinizing cells in culture, suggesting the existence of a defect in differentiation unrelated to vitamin A.

A study with a small cell lung cancer cell line (Lu-134-B-S) revealed that the small cell phenotype was maintained only when cultured in the presence of regular fetal calf serum. When cultured in delipidized serum, the cells became squamous, as indicated by synthesis of involucrin and high molecular weight keratins, as well as the appearance of desmosomes. The squamous phenotype reverted to the small cell phenotype when retinoic acid was added to cells maintained in delipidized serum.

We observed that HNSCC 1483 cells grown in delipidized serum depleted of endogenous retinoids expressed keratins with molecular weights of 67 (K1), 56 (K10), 54, 52, 48, 46, and 40 (K19) kD [22]. In contrast, cells grown in medium with 10% fetal bovine serum containing about 0.06 μM retinol expressed much less K1; the levels of keratins with molecular weights of 46 and 48 kD were also lower, but the amounts of the 52 and 40 kD keratins were higher than those expressed in cells grown in delipidized serum. Cells treated with tRA in delipidized serum contained lower levels of keratins with molecular weights of 67, 56, 54, 48, and 46 kD and higher levels of the 52 kD (probably K8) and the 40 kD (probably K19) keratins than cells grown without retinoic acid. Thus, tRA modulated the expression of several keratins in addition to K1 in HNSCC 1483 cells [22]. These results are similar to the previous finding that normal keratinocytes and cells from SCCs of human tongue and skin produce keratins larger than 60 kD, including K1, when floated on collagen rafts, or grown in delipidized serum, or on de-epidermized dermis at the air-liquid interface [6]. Laryngeal epithelial cells and papilloma cells respond to retinoids as demonstrated in a recent study of cells cultured *in vitro* at the air-liquid interface. tRA was found to modulate the differentiation of these cells along two distinct pathways. At low concentrations ($<10^{-8}$ M) the cells formed stratified squamous epithelium and expressed K13; at higher concentrations of tRA ($>10^{-7}$ M) the cells differentiated into a columnar epithelium with occasional ciliated cells, lacking squamous markers [27].

Retinoids also inhibited the synthesis of loricrin and profilaggrin; the conversion of profilaggren to filaggrin in cultured human epidermal keratinocytes [28]; and the production of cholesterol sulfate in rabbit tracheal epithelial cells, human bronchial epithelial cells, human keratinocytes, and in several HNSCC cell lines. The mechanism appears to be suppression of the cholesterol sulfotransferase activity by retinoids [14]. Likewise, retinoids suppressed the levels of lipids (acylceramide and lanosterol) that normally increase during the late stages of epidermal differentiation, and the expression of a preprorelaxin-like gene in rabbit and human tracheobronchial epithelial cells and in human keratinocytes.

Recent histologic and immunocytochemical analyses of the effects of topically applied tRA on the expression of squamous differentiation markers in human skin *in vivo* revealed some differences from published findings with cultured epidermal keratinocytes *in vitro* [29]. tRA treatment *in vivo* (either an acute 4-day, or a chronic 16-week treatment) caused epidermal thickening, stratum granulosum thickening, and increases in the number of cell layers expressing TGase-I, involucrin, and filaggrin, as well as focal expression of keratins K6 and K13, which are not expressed in normal epidermis. The acute tRA treatment decreased loricrin levels whereas the chronically treated skin showed increased number of cell layers expressing loricrin. The *in vivo* treatment did not alter the expression of keratins K1, K10, and K14 [29]. A direct comparison of acutely *in vivo*-treated skin and cultured keratinocytes exposed to tRA *in vitro*, revealed that TGase-I activity increased in the skin but decreased in the cultured cells [30]. These results demonstrate clearly that some of the results obtained with cultured normal cells *in vitro* may not represent the response of cells *in vivo*. It should be noted that the topical treatment used 0.1% tRA (1 mg/ml in cream), whereas the cultured cells were exposed to a much lower concentration of tRA (0.3 μg/ml; 1 μM).

MECHANISMS INVOLVED IN THE ACTIONS OF RETINOIDS AND THE ROLE OF NUCLEAR RETINOID RECEPTORS

Although the ability of retinoids to inhibit proliferation and clonogenicity of malignant cells and to modulate their differentiation *in vitro* is well documented, the mechanism(s) responsible for these effects is not fully understood. The modulation of gene expression is the most plausible mechanism by which retinoids effect the differentation and growth of malignant cells, or suppress the progression of premalignant cells to frank neoplastic lesions. The identity of the genes that control the expression of the premalignant or the malignant phenotype is not known; however, the restoration of normal differentiation by retinoids may represent a part of a retinoid-dependent program of gene expression that includes activation of intrinsic anticancer activity (*e.g.*, suppressor genes) or inhibition of genes that maintain the malignant phenotype in HNSCC cells. In the case of leukoplakia, retinoids may activate a program of resistance to neoplastic progression.

To modulate gene expression, retinoids must transmit signals to the cell nucleus. The signal transduction mechanism is beginning to be unravelled as understanding of the roles of the major pathway components increases. Both cytoplasmic and nuclear retinoid-binding proteins appear to play important roles in the series of events initiated by the uptake of retinoids by target cells, culminating in the modulation of gene transcription in the cell's nucleus.

The expression of nuclear retinoic acid receptors (RARs) in various embryonal tissues and in keratinizing and nonkeratinizing epithelial tissues and in skin and oral mucosal cells has been analyzed by Northern blotting or by *in situ* hybridization. RAR-γ is the predominant receptor in skin. RAR-α is expressed at much lower levels than RAR-γ, and RAR-β mRNA is undetectable. A similar pattern of receptor expression is also found in RA-treated skin. RAR-γ1 mRNA is localized to all layers of the epidermis including the basal cells.

Cultured undifferentiated tracheobronchial epithelial cells from human (HBE) and rabbit (RbTE) express both RAR-α and RAR-γ constitutively; the expression of these receptors is not altered after the cells undergo squamous cell differentiation or after RA treatment [31]. In contrast, the constitutive RAR-β mRNA level, which is low in HBE and RbTE, increased several-fold after RA treatment of both cell types [31]. All but 1 of 9 surgical specimens of "normal" lung tissue adjacent to lung carcinomas expressed RAR-β when analyzed by Northern blotting, indicating that this receptor is constitutively expressed *in vivo* [32]. However, the method used was not appropriate to indicate whether the RAR-β is present in the epithelial cells, the stromal cells, or both.

The expression of RAR-α and RAR-γ mRNA was detected in all cell strains derived from normal oral mucosa [33,34]. In contrast, RAR-β mRNA was detected only in cell lines derived from nonkeratinizing soft palate and the floor of the mouth. It was not detectable in nonkeratinizing buccal mucosa or in the keratinizing epithelial cells from the hard palate and gingiva [33,34]. RA treatment increased RAR-β mRNA levels in all 3 nonkeratinizing cell lines but not in the keratinizing ones. These results suggested that RAR-β expression is inversely related to keratinization.

Cell lines derived from oral leukoplakias from different regions of the oral cavity expressed RAR-α and RAR-γ constitutively, but the level of RAR-γ was about one half that of normal epithelial cells derived from the corresponding region of the oral cavity [33,34]. In contrast, only those cells derived from leukoplakias of the soft palate expressed RAR-β [33,34]. Treatment with RA increased RAR-β level in the cells that expressed it constitutively, but not in cells that did not express it prior to treatment [33,34]. Our analysis by *in situ* hybridization of surgical specimens from oral leukoplakic lesions revealed that RAR-β was present in 4 of 6 tongue specimens, but was not detected in any of the 4 buccal mucosa specimens.

SCC cell lines derived from cancers of the oral cavity expressed RAR-α and RAR-γ, with RAR-γ being lower in 6 of 9 SCCs relative to their normal counterparts [34]. RAR-β was expressed in only 2 of 7 HNSCC cell lines. A loss of expression relative to normal was evident in 2 soft palate SCCs and 1 floor of the mouth tumor [34]. These results raised the possibility that an abnormally low level of RAR-β may contribute to neoplastic progression in stratified squamous epithelia [34]. We analyzed the ex-

pression of 6 nuclear retinoic acid receptors (RAR-α, RAR-β, RAR-γ, and RXR-α, RXR-β, and RXR-γ) in 4 HNSCC cell lines derived from tonsil (HNSCC183), larynx (HNSCC886Ln), retromolar trigon (HNSCC1483), and buccal mucosa (SqCC/Y1), grown in the absence or presence of retinoic acid [35]. All 4 cell lines expressed mRNAs for RAR-α, RAR-γ, and RXR-α; 3 cell lines (183, 886, and 1483) expressed RAR-β; and none expressed RXR-β or -γ. SqCC/Y1 did not express RAR-β as was reported earlier [36]. tRA treatment increased the level of RAR-β in the cell lines that expressed it constitutively but not in the one (SqCC/Y1) that did not express it. In contrast, the treatment had little or no effect on the expression of RAR-α or RXR-α.

To assess the expression of nuclear RA receptors *in vivo*, we used digoxigenin-labeled cRNA probes of RAR-α and RAR-β, and RAR-γ antisense for *in situ* hybridization to sections of head and neck surgical specimens including normal, hyperplastic, dysplastic, premalignant (*e.g.*, leukoplakia) and malignant (SCC) tissues. An analysis of 31 head and neck tissue specimens including 14 oral cavity and 17 pharyngeal and laryngeal cases revealed that RAR-α and RAR-γ mRNAs were present in most of the tissue specimens at similar levels. In contrast, RAR-β, which was detected in 70% of the normal and hyperplastic lesions, was detected in only 56% of the dysplastic lesions and 35% of the carcinomas [37]. These results strongly indicate that decreased expression of RAR-β may be associated with the development of head and neck cancer.

The expression of nuclear RA receptors in human lung SCCs was reported independently by three laboratories. Their results were based on Northern blotting and showed that most squamous and adenosquamous carcinomas expressed RAR-α and RAR-γ mRNAs. The level of RAR-γ was higher in 3 of 7 squamous carcinomas than in other cell lines and normal lung [32]. The expression of RAR-β was variable. It was expressed by 3 of 8 SCCs and was induced by RA in 4 of 8 cell lines. RAR-β mRNA was expressed in 5 of 7 adenosquamous carcinomas and was increased by RA treatment in 2 of 7 of these cell lines [31,32,38]. Interestingly, RAR-β abnormalities were also observed in small cell lung carcinomas as 3 of 11 cell lines failed to express RAR-β mRNA [32]. RAR-β DNA showed rearrangements in one SCC that did not express RAR-β as well as in an adenocarcinoma and small cell carcinoma that did express RAR-β [32]. Northern blot analyses of RAR-β mRNA in surgical specimens of adjacent "normal" and lung carcinomas showed that 3 of 9 tumor samples contained no or low transcript levels relative to normal tissue [32]. The abnormalities in RAR-β expression suggested that this receptor may be involved in the pathogenesis of lung cancer, possibly as a suppressor gene [32,38]. However, no rearrangements of RAR-β gene were detected in several surgical specimens of lung cancer [32], and the expression of thyroid hormone receptor (located close to RAR-β on chromosome 3p24 in cell lines that did not express RAR-β), indicated that the RAR-β gene was not deleted in the lung carcinoma cells [38]. Thus, the molecular mechanism underlying the relationship between aberrant expression of RAR-β and the development of lung cancer is still unknown.

Retinoid regulation of gene expression in keratinocytes is thought to occur at the transcriptional level [39,40]. Nuclear RARs have been implicated in the regulation of epidermal keratinocyte differentiation, based on the correlation between the ability of several synthetic retinoids to both bind to RARs and transactivate transcription from retinoic acid receptor element (RAR-E), and to suppress the expression of squamous differentiation markers such as involucrin, TGase-I, and cornifin (SQ37) in normal human epidermal keratinocytes [36]. A role for nuclear RA receptors in the regulation of squamous differentiation has been demonstrated more directly for keratin genes [40]. The three RAR receptor subtypes were able to suppress transcription from the 5′ regulatory regions of keratins K5, K6, K10, and K14 in cotransfection experiments [40]. Although direct binding of the RARs to DNA was not demonstrated, it was proposed that the RARs bind to putative negative recognition elements in the upstream DNAs of the keratin genes [40]. Roles for nuclear RA receptors in both positive and negative regulation of epidermal differentiation have been implied by the effects of transfecting a truncated RAR-γ receptor [41]. RAR-γ truncated in the ligand-binding domain enhanced the growth and inhibited the squamous differ-

entiation (*e.g.*, production of keratins K1/10 and K6/16, involucrin, and filaggrin) of human SCC13. In addition, the transfected cells expressed low levels of K13 and K19, which are positively regulated by retinoids in the parental SCC13 cells. Thus, it appears that RARs may be required to induce terminal differentiation of keratinocytes [41]. RAR-β mRNA expression in cultured normal oral epithelial cell strains was correlated with the expression of keratin 19; it was suggested that RAR-β plays a role in keratin expression and suprabasal differentiation of stratified squamous epithelia [33]. This correlation was abrogated in HNSCCs where the expression of RAR-β was independent of the expression of K19. Likewise, there was no apparent correlation between the expression of the nuclear receptors and the status of squamous cell differentiation of the HNSCC cell lines (2 well-differentiated and 2 poorly-differentiated). There was no correlation between the expression of RAR-β and the response of HNSCC cells to the growth inhibitory or differentiation-suppressing effects of tRA [35].

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