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INHIBITION OF GROWTH AND SQUAMOUS-CELL DIFFERENTIATION MARKERS IN CULTURED HUMAN HEAD AND NECK SQUAMOUS CARCINOMA CELLS BY β-ALL-TRANS RETINOIC ACID

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Vitamin A and some of its metabolites such as β-all-trans retinoic acid (RA) have been implicated in the regulation of differentiation of normal and malignant epithelial cells *in vivo* and *in vitro*. In the present study the effects of RA on the growth and differentiation of 7 cell lines derived from human head and neck squamous-cell carcinomas (HNSCCs) were examined. RA (>0.01 μM) inhibited the proliferation in monolayer culture of 6 of 7 HNSCC cell lines. One cell line (UMSCC-35) was very sensitive, 5 (UMSCC-10A, -19, -30, -22B and HNSCC 1483) were moderately sensitive, and 1 (HNSCC 183) was insensitive. Three of the cell lines (UMSCC-22B, -30, and HNSCC 1483) were capable of forming colonies in semi-solid medium—a capability that was suppressed by RA. The HNSCC cell lines expressed various levels of the squamous-cell differentiation markers type I (particulate, epidermal) transglutaminase (TGase) and cholesterol sulfate (CS). RA treatment (1 μM, 6 days) decreased TGase activity by more than 50% in 3 (UMSCC-10A, -22B and 1483) of the 7 cell lines, and the effect on UMSCC-22B was dose-dependent. Type II TGase (soluble, tissue type) activity was detected in 3 cell lines, and after RA treatment its activity increased in HNSCC 1483 and 183 cells and decreased in UMSCC-19. Following RA treatment, CS levels decreased by 20, 25, 70, 76, 89 and 91% in cell lines UMSCC-30, -10A, 183, UMSCC-35, -22B, and HNSCC 1483, respectively. The suppression by RA of CS accumulation in the 1483 cells was dose-dependent. Cholesterol sulfotransferase activity, which is responsible for CS synthesis, was suppressed by 40–97% after RA treatment of UMSCC-19, -22B, and HNSCC 1483. Our results demonstrate that RA inhibits the growth and decreases the level of 2 squamous differentiation markers in HNSCC cells.

The proper differentiation of epithelial cells, including those of the oral cavity and upper aerodigestive tract, depends on adequate vitamin A. Vitamin A deficiency is accompanied by a replacement of mucociliary epithelial cells by squamous cells and this squamous metaplasia can be reversed by supplementation with vitamin A or some of its analogs (retinoids) (Wolbach and Howe, 1925; Wolbach, 1956; Chopra, 1983; McDowell *et al.*, 1984). Experimental oral carcinogenesis is enhanced by vitamin A deficiency (Rowe and Gorlin, 1959), and pre-neoplastic changes induced by chemical carcinogens, which often lead to squamous metaplasia, can be suppressed by retinoids (Shklar *et al.*, 1980; Burge-Bottenbley and Shklar, 1983).

Squamous carcinomas account for nearly 90% of the cancers in the oral cavity (Shafer *et al.*, 1974), and there is ample evidence that retinoids can suppress the development of oral squamous carcinomas in experimental animals exposed to various carcinogens (Shklar *et al.*, 1980; Burge-Bottenbley and Shklar, 1983) and in patients with leukoplakia, a pre-malignant lesion of the oral cavity (Hong *et al.*, 1986 and references therein). These and other results have led to pre-clinical and clinical trials designed to explore the potential of using retinoids for prevention and treatment of various types of human cancer (Bertram *et al.*, 1987; Lippman *et al.*, 1987).

Numerous reports have described the effects of retinoids on the growth and differentiation of normal, pre-malignant and malignant cells in tissue explants, cell cultures and established cell lines (Jetten, 1987; Lippman *et al.*, 1987; Lotan, 1980; Roberts and Sporn, 1984). Particularly extensive and informative were investigations of cells derived from epithelial tissues (*e.g.*, keratinocytes and tracheo-bronchial epithelial cells) and carcinomas. Such studies have shown that retinoids modulate the morphology and growth of epithelial cells and tissues (Asselineau *et al.*, 1989; Chopra and Flaxman, 1975; Fitzgerald *et al.*, 1986; Hashimoto *et al.*, 1985; Kubilus *et al.*, 1981; McDowell *et al.*, 1987; New, 1963; Reiss *et al.*, 1985), alter keratin gene expression (Fuchs and Green, 1981; Gilfix and Eckert, 1985; Huang *et al.*, 1986; Jetten *et al.*, 1989*c*; Kim *et al.*, 1984; Rubin *et al.*, 1989; Tseng *et al.*, 1984; Wu and Wu, 1986), and suppress the expression of squamous-cell differentiation markers including type I (epidermal) transglutaminase (TGase) (Jetten and Shirley, 1986; Jetten *et al.*, 1987, 1989 *a,b*; Lichti and Yuspa, 1988; Lichti *et al.*, 1985; Parenteau *et al.*, 1986; Rubin and Rice, 1986; Rubin *et al.*, 1989; Thacher *et al.*, 1985), involucrin (Cline and Rice, 1983; Reiss *et al.*, 1985; Rubin *et al.*, 1989), envelope crosslinking (Green and Watt, 1982; Ke *et al.*, 1988; Nagae *et al.*, 1987; Rearick *et al.*, 1988; Yaar *et al.*, 1981), and cholesterol sulfate (CS) level (Rearick and Jetten, 1986; Rearick *et al.*, 1987*a,b*, 1988).

Being interested specifically in the application of retinoids for prevention or treatment of oral cancer, we are examining the responsiveness of cultured HNSCC cells to retinoids. In a previous study of 2 HNSCC lines, we found that RA inhibited the growth of 1 cell line designated HNSCC 1483 but failed to affect the growth of another cell line, HNSCC 183 (Lotan *et al.*, 1987). More recently, we have found that RA inhibits the growth of multicellular tumor spheroids formed by a human HNSCC MDA 886Ln cell line and suppresses involucrin production (Sacks *et al.*, 1989). Since malignant transformation of epithelial cells of the upper aerodigestive tract is often accompanied by aberrant squamous-cell differentiation and the majority of cancers of the head and neck are squamous-cell carcinomas, we examined in the present study the effects of retinoids on cell growth and on the expression of squamousdifferentiation markers in 7 HNSCCs.

MATERIAL AND METHODS

Cell culture and RA treatment procedures

Details of the patients and the location within their oral

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Abbreviations: CS, cholesterol sulfate; HEPES, [4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid]; HNSCC, head and neck squamous-cell carcinoma; PBS, phosphate-buffered saline; RA, retinoic acid; TGase, transglutaminase.

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cavity of the squamous-cell carcinomas from which the HNSCC cell lines used were derived are presented in Table I. These cells were grown in monolayer culture or in semi-solid agarose (0.5%) and treated with RA exactly as described by Lotan *et al.* (1987). The medium contained about 1.7 mM Ca^{2+}.

Assay of type-I and type-II TGase activities

Monolayers of cells, grown on plastic tissue culture dishes (10 cm in diameter), were washed, frozen, thawed and scraped in PBS supplemented with 10 mM dithiothreitol. The cells were then sonicated and centrifuged at 100,000 *g*. Triton X-100 was added to the supernatant (containing the soluble type-II TGase) and to the pellet (particulate fraction containing the type-I TGase) to a final concentration of 1%, and TGase activity in both fractions was determined by measuring their ability to attach covalently [^{3}H]putrescine (18.6 Ci/mmol, Dupont, Boston, MA) to casein during a 20-min incubation. Additional details of the assay were described earlier (Jetten and Shirley, 1986).

CS assay

The method described by Rearick and Jetten (1986) was used to determine CS level. Briefly, cells were labelled during the last 24 hr of growth by including $Na_2[^{35}S]O_4$ (50 μCi/ml; carrier-free, ICN, Irvine, CA) in the medium using 4 ml of medium/10-cm diameter dish. The cell monolayers were then washed twice in PBS, detached by brief trypsinization and centrifuged, then the pellets were frozen at −70°C. The pellets were thawed, suspended in 4 ml of chloroform:methanol (2:1, vol:vol), sonicated, and centrifuged. The supernatant was partitioned by adding 1 ml of 0.1 M KCl and mixing vigorously. After centrifugation the upper aqueous phase was removed, and the lower phase was re-extracted with 2 ml of methanol:0.1 M KCl (1:1). After centrifugation the organic phase was dried and the radioactivity was counted.

Assay of cholesterol sulfotransferase activity

Cells grown as monolayers on plastic dishes were scraped off the dishes in 50 mM HEPES buffer, pH 7.3, and sonicated. The cholesterol sulfotransferase activity was determined in the cell extracts by measuring their ability to transfer [^{35}S]sulfate from 3′-phosphoadenosine-5′ phospho-[^{35}S]sulfate to cholesterol (Rearick *et al.*, 1987).

RESULTS

Effect of RA on anchorage-dependent and anchorage-independent growth of HNSCC cells

The growth of 6 of the 7 HNSCC cell lines in monolayer culture was inhibited to various degrees and in a dose-dependent fashion by RA (Fig. 1*a*). UMSCC-35 was the most sensitive cell line, exhibiting 90% growth inhibition at 0.1 μM RA. In contrast, cell line 183 was only marginally affected, showing less than 20% inhibition after treatment with 1 μM RA. The growth of cell lines 1483 and UMSCC-22B, -19, -10A, and -30 was inhibited by 40–60% after treatment with 1 μM RA. The inhibitory effect was apparently cytostatic rather

TABLE I – SOURCE OF THE HNSCC CELL LINES

Cell line	Location of tumor	Patient[4]
1483[1]	Retromolar trigone	M/66/C
183[1]	Tonsil	M/54/C
UMSCC-10A[2]	Larynx	M/57/C
UMSCC-19[2]	Base of tongue	M/67/C
UMSCC-22B[2]	Lymph node[3]	M/53/C
UMSCC-30[2]	Pyriform sinus	F/56/B
UMSCC-35[2]	Oropharynx	M/51/B

[1]Sacks *et al.*, 1988.–[2]Cells obtained in 1985 from Dr. T. Carey (Univ. of Michigan, Ann Arbor, MI). The derivation and properties of some of them were described previously (Krause *et al.*, 1981).–[3]Metastasis from hypopharynx.–[4]C, Caucasian; B, black.

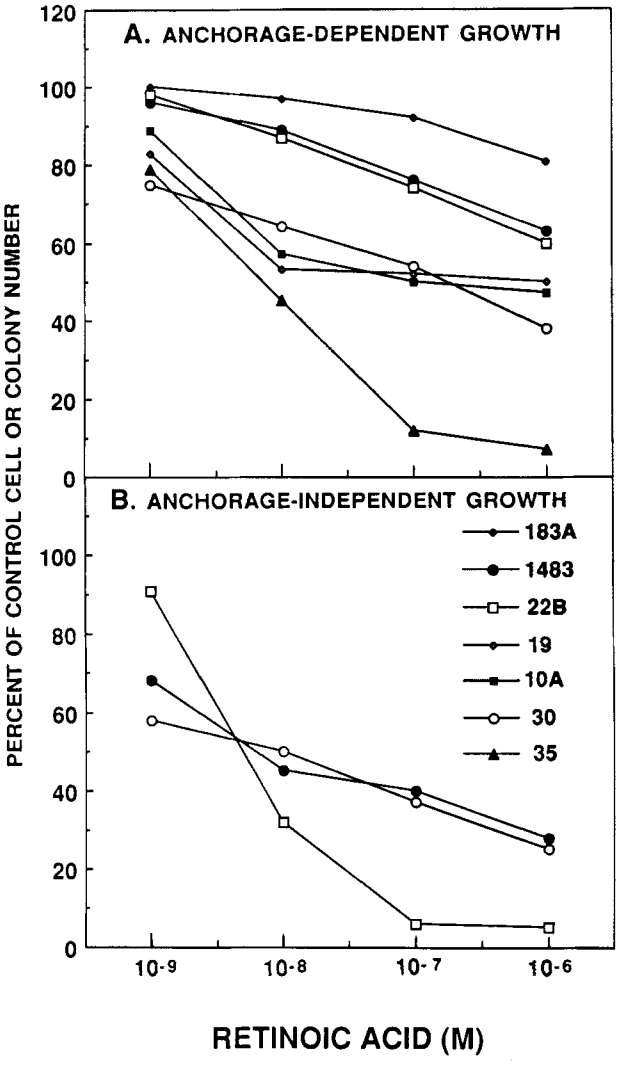
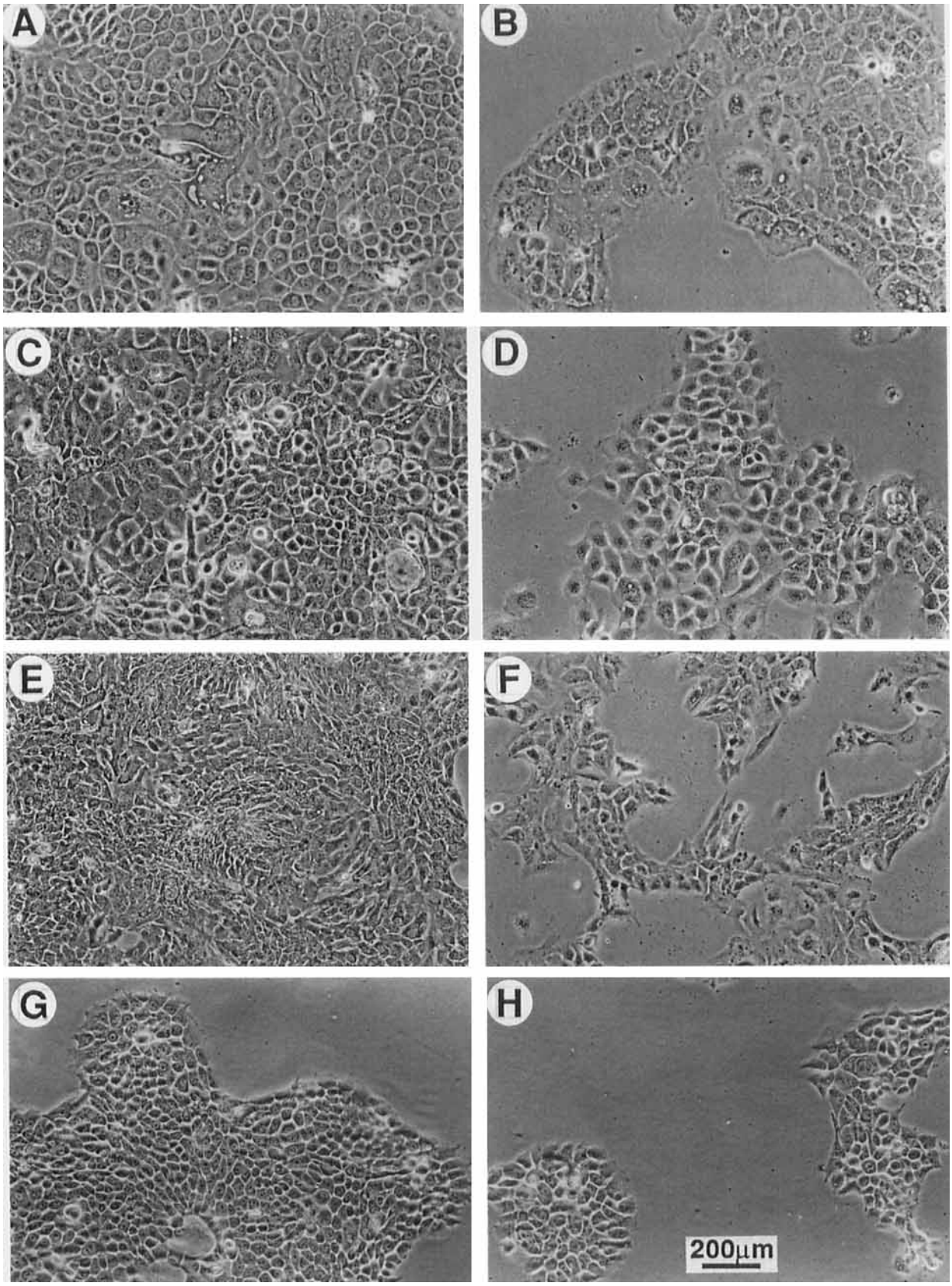


FIGURE 1 – Inhibition by RA of HNSCC cell growth. *(a)* Cells were seeded at 2 × 10^4/dish in a series of 35-mm dishes. After 24 hr of incubation the medium was replaced by one containing either 0.01% DMSO or the indicated RA concentrations obtained by dilution of RA from a 10-mM stock solution in DMSO. The cells were fed fresh medium every 72 hr and detached and counted after 12 days. *(b)* Cells were suspended at 10^5 cells/ml in 0.5% agarose in medium containing either DMSO (0.01%) or one of the indicated RA concentrations. One-milliliter samples were placed on top of a pre-cast and solidified layer of 1% agarose. The cell-containing agarose was allowed to gel at 4°C and then the cells were incubated at 37°C. After 72 hr 1 ml of fresh medium with or without RA was placed on top of the agarose and replaced with fresh medium every 72 hr. After 2 weeks the number of colonies was determined under an inverted microscope.

FIGURE 2 – Morphology of HNSCC cells. Cells were grown on plastic tissue culture dishes in the absence (*a, c, e* and *g*) or presence of 1 μM RA (*b, d, f* and *h*) for 12 days and then photographed under a phase-contrast microscope. *(a)* and *(b)* UMSCC-10A; *(c)* and *(d)* UMSCC-19; *(e)* and *(f)* UMSCC-35; *(g)* and *(h)* 1483.


A
B
C
D
E
F
G
H
200µm

than cytotoxic since cell viability after treatment with 1 μM RA was greater than 95% (not shown). The appearance of some of the HNSCC cell lines under the phase-contrast microscope is shown in Figure 2. The morphology of treated cells is not markedly changed; however, treated cells appear to be somewhat more flattened and better spread on the substrate than untreated cells.

Three of the HNSCC cell lines (1483, UMSCC-22B and UMSCC-30) were capable of forming colonies (>60 μm in diameter) in agarose and this ability was suppressed by RA (Fig. 1*b*). The RA dose required for 50% inhibition of colony formation was similar (about 4 nM) for the 3 cell lines. However, at higher doses the UMSCC-22B cells were more sensitive to RA suppression of anchorage-independent growth than the 2 other cell lines. HNSCC 183 cells formed only small colonies (<25 μm in diameter), and their formation was also inhibited by RA (data not shown but see Lotan *et al.*, 1987).

Effect of RA on type-I TGase activity

The 7 HNSCC cell lines expressed different levels of type-I TGase (Fig. 3, empty bars). The highest level (47,000 cpm/20 min/mg protein) was detected in the 1483 cells; cell lines 183, UMSCC-10A, -19, and -22B contained considerably lower enzyme levels (4,600–8,900 cpm/20 min/mg protein), whereas the UMSCC-30 and -35 contained very low TGase levels. RA treatment exerted various effects on TGase activity, decreasing it in the 1483, UMSCC-10A and -22B cells by 50–75%, and affecting it only marginally in the 183, UMSCC-19, -30 and -35 cells (Fig. 3). The inhibition of TGase in the UMSCC-22B cells was dependent on RA concentration, with 50% inhibition occurring at about 1 nM RA (Fig. 4).

Effect of RA on type-II TGase activity

The 7 HNSCC cell lines expressed different levels of type-II TGase (Fig. 5, empty bars). The highest level (40,150 cpm/20 min/mg protein) was detected in the UMSCC-19 cells; cell line 183 contained about half as much TGase activity, and the level in the 1483, UMSCC-10A, and -22B cells was very low. No activity was detected in the UMSCC-30 or -35 cells. RA treatment decreased TGase activity by 50% in the UMSCC-19 cells and increased it in the 1483 and the 183 cells (Fig. 5). There was no increase in TGase type-II in the UMSCC-30 and -35 cells.

Effect of RA on CS level and on cholesterol sulfotransferase activity

The 7 HNSCC cell lines expressed different CS levels ranging from 500 in the UMSCC-30 cells to 8,000 cpm/mg protein in the UMSCC-10A and -35 cells (Fig. 6). RA treatment decreased the CS level in 5 of the 7 cell lines and increased it in the UMSCC-19 cells (Fig. 6). The decrease in CS in the 1483 cells was dose-dependent, and 50% inhibition occurred at 1 nM RA (Fig. 7).

Cholesterol sulfotransferase activity was high in the cells expressing high CS levels (1483 and UMSCC-22B) and low in those expressing low CS levels (UMSCC-19 and -30) (Table II). However, there was no linear relationship between the level of cholesterol sulfotransferase and CS. This may be due to the dependence of CS level not only on the level of the biosynthetic enzyme, but also on the rate of degradation. RA treatment decreased the level of the cholesterol sulfotransferase by 40–97%. This effect can explain, at least in part, the decreased CS level in RA-treated cells.

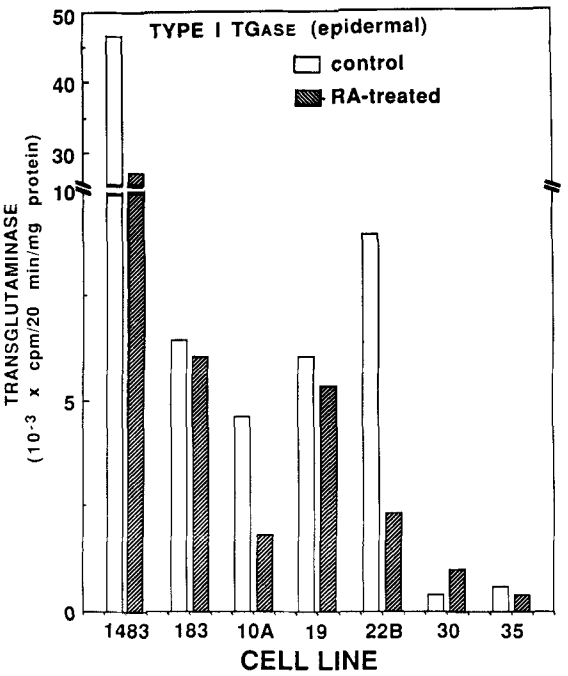


FIGURE 3 – Effect of RA on type-I TGase in HNSCC cells. Cells were cultured in the absence or presence of 1 μM RA. The medium was replaced after 72 hr and at daily intervals thereafter. After 6 days the cell monolayers were washed twice in PBS, pH 7.2 and frozen at –70°C. The particulate, type-I TGase activity was then analyzed as described in "Material and Methods." Duplicate dishes were used for the assays, and each assay was performed in duplicate or triplicate. SE was <10%.

DISCUSSION

The present study has shown that RA can suppress cell growth and the level of squamous differentiation markers in cultured HNSCC cells (Table III). Though malignant and normal epithelial cells may share similar physiological or pharmacological responses to retinoids, certain malignant cells may possess aberrant growth and differentiation control mechanisms that alter their response to retinoids. There are several reports on the effect of retinoids on the growth of normal human keratinocytes (Chopra and Flaxman, 1975; Hashimoto *et al.*, 1985; Kubilus *et al.*, 1981). Outgrowth of epithelial cells from normal human skin in the absence of underlying connective tissue was stimulated by retinol (Chopra and Flaxman, 1975). However, the plating efficiency of human keratinocytes derived from infant foreskins on mitomycin C-treated 3T3-Swiss fibroblasts was reduced in the presence of RA or retinyl acetate at 3 μM or higher concentrations (Kubilus *et al.*, 1981). There was no growth inhibition of cells that were allowed to establish colonies before being exposed to retinoids, and the viability of cells in the treated cultures was prolonged compared to the viability in the untreated ones. In contrast, retinol, RA, and several other retinoids inhibited the growth of cultured human epidermal cells on a feeder layer of 3T3 mouse fibroblasts in medium containing low (0.2 mM) Ca^{2+} concentration, which enabled epidermal cells to grow rapidly without stratification (Hashimoto *et al.*, 1985). The treated epidermal cells also exhibited reduced spreading on the substrate.

Different malignant keratinocytes also exhibit diverse responses to retinoid treatment. Whereas the colony-forming efficiency on plastic substrate by SCC-13 cells, derived from a facial skin carcinoma, decreased after prolonged retinyl acetate treatment (Cline and Rice, 1983), the colony-forming efficiency of SqCC/Y1 buccal mucosa SCC was stimulated by pre-treatment with RA (Reiss *et al.*, 1985). The growth of 3 cell lines derived from SCC of the facial skin (SCC-13, SCC-

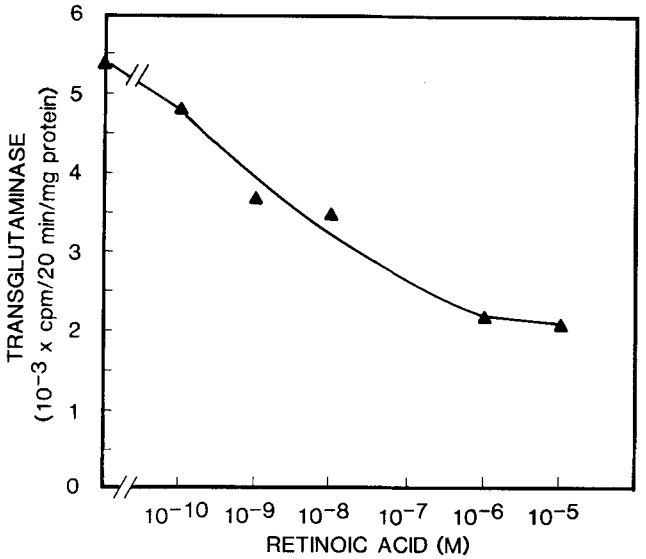


FIGURE 4 – RA dose-dependent suppression of type-I TGase activity in UMSCC-22B cells. Cells were cultured for 6 days in the absence or presence of the indicated RA concentrations, washed twice in PBS, pH 7.2, and frozen at –70°C. The activity of the particulate type-I TGase in the cells was determined as described in Figure 3.

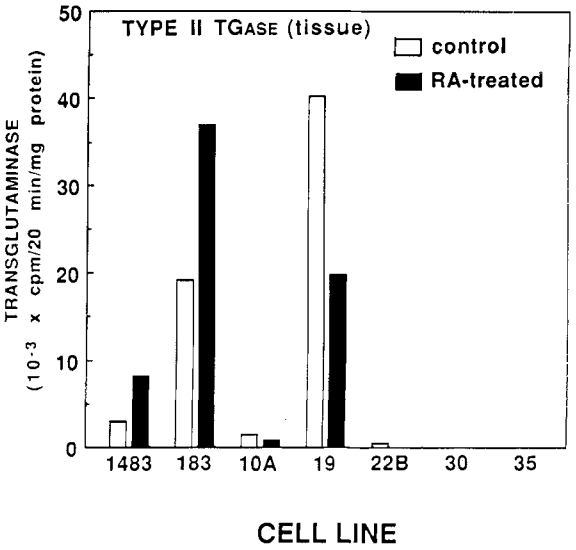


FIGURE 5 – Effect of RA on type-II TGase in HNSCC cells. Cells were cultured in the absence or presence of 1 μM RA. The medium was replaced after 72 hr and at daily intervals thereafter. After 6 days the cell monolayers were washed twice in PBS, pH 7.2, and frozen at –70°C. The activity of soluble, type-II TGase was then analyzed as described in "Material and Methods." Duplicate dishes were used for the assays, and each assay was performed in duplicate or triplicate. SE was <10%.

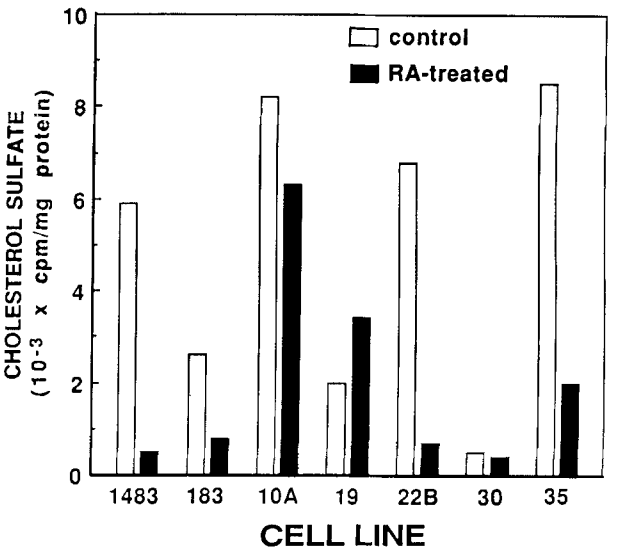


FIGURE 6 – Effect of RA on CS levels in HNSCC cells. Cells were cultured in 10-cm diameter dishes in the absence or presence of 1 μM RA for 6 days. The medium was replaced after 72 hr and then daily. During the last 24 hr of incubation, the medium contained $Na_2[^{35}S]O_4$. On day 6 the amount of CS was determined as described in "Material and Methods." Thin-layer chromatography revealed that the radioactivity in this material co-chromatographed with CS (not shown).

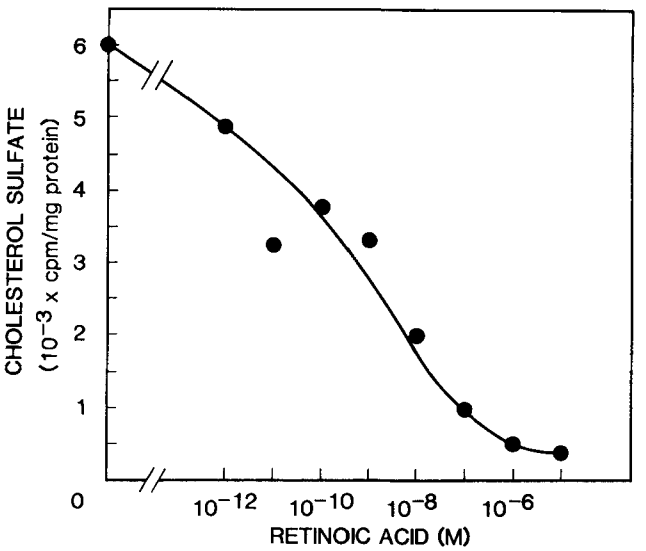


FIGURE 7 – RA dose-dependent suppression of CS levels in 1483 HNSCC cells. Cells were cultured in the absence or in the presence of the indicated RA concentrations. The medium was replaced after 72 hr and then daily up to 6 days. The CS level in the cells was determined as in Figure 5.

12B2 and SCC-12F2) and 2 derived from SCC of the tongue (SCC-4 and SCC-9) was affected differently after a week's treatment of confluent cultures with 3.3 μM RA in medium containing 1.8 mM Ca^{2+}. Cell lines SCC-4, SCC-9 and SCC-13 exhibited 17%, 39% and 44% growth inhibition, whereas the growth of SCC-12B2 was not affected (Rubin and Rice, 1986). An apparent increase in the growth of SCC-12F2 was probably due to desquamation of cells in untreated cultures. Here we have demonstrated that RA inhibits the growth of 6 of 7 HNSCC cell lines in monolayer culture in medium containing 1.7 mM Ca^{2+} without causing marked changes in cell morphology. Cells treated with 1 μM RA were inhibited by 35–95% after a 12-day treatment.

A property characteristic of transformed and tumor cells and not expressed by most normal and untransformed cells is the ability to form colonies in semi-solid medium such as agar or agarose (Neugut and Weinstein, 1979). Successful clonal growth in agar of cells derived directly from fresh human HNSCCs was achieved in 56–64% of samples of primary and metastatic HNSCC lesions (Mattox and Von Hoff, 1980; Schiff

TABLE II – EFFECT OF RETINOIC ACID ON CHOLESTEROL SULFOTRANSFERASE ACTIVITY IN HNSCC CELLS

Cell line	Cholesterol sulfotransferase activity (pmol/hr/μg protein)	
	Control	RA-treated
1483	985	224
UMSCC-19	27	16.2
UMSCC-22B	268	6.4
UMSCC-30	25.7	5.7

Cells were cultured for 6 days in the absence or presence of 1 μM RA. Cholesterol sulfotransferase activity was determined as described in "Material and Methods."

TABLE III – SUMMARY OF RETINOIC ACID EFFECTS ON HNSCC

Cell line	Growth		Squamous differentiation marker	
	Monolayer	Agarose	Type-I TGase	Cholesterol sulfate
1483	↓	↓	↓	↓
183	=	NA	=	↓
UMSCC-10A	↓	NA	↓	↓
UMSCC-19	↓	NA	=	↑
UMSCC-22B	↓	↓	↓	↓
UMSCC-30	↓	↓	↑[1]	=[1]
UMSCC-35	↓	NA	=[1]	↓

=, no change; NA, not applicable because the untreated cells did not form colonies in agarose.–[1]The level of this marker was very low before or after RA treatment. Therefore, the changes induced by RA may have no biological significance.

and Shugar, 1984). The colony-forming efficiency in agar was quite low and ranged from 0.001 to 0.08% (Schiff and Shugar, 1984). By replacing agar with agarose a 2-fold increase in cloning efficiency was achieved (Schiff and Shugar, 1984). Both poorly-differentiated and moderately-differentiated squamous-cell carcinomas had higher success rates than well-differentiated ones; however, high and low cloning efficiencies were found in each histologic group (Schiff and Shugar, 1984). The colony-forming efficiency in agar or agarose of cell lines established from HNSCCs is also very low (<0.02%) (Rupniak *et al.*, 1985). In the present study, 3 of the 7 HNSCC cell lines were capable of forming colonies in agarose, but this anchorage-independent growth was suppressed in the presence of RA. Such an effect has been observed previously with cells other than HNSCC and is thought to represent suppression by RA of the expression of the transformed phenotype (Lotan, 1980; Roberts and Sporn, 1984).

Previous studies using rodent and human keratinocytes and rabbit and human tracheobronchial epithelial cells have established that squamous-cell differentiation involves increases in type-I (particulate) TGase (Jetten and Shirley, 1986; Thacher, 1989; Thacher and Rice, 1985), the calcium-dependent membrane-associated enzyme responsible for cross-linking involucrin and other proteins to form a cross-linked envelope (Michel *et al.*, 1987; Simon and Green, 1985; Thacher and Rice, 1985), and in the level of cholesterol 3-sulfate (Jetten, 1987; Rearick and Jetten, 1986; Rearick *et al.*, 1987*a,b*, 1988). The ability to express type-I TGase and to form cross-linked envelopes is retained by some squamous-cell carcinomas (Reiss *et al.*, 1985; Rubin and Rice, 1986; Thacher, 1989; Thacher and Rice, 1985). Likewise, some carcinoma cells express CS (Rearick *et al.*, 1988).

Malignant transformation of epithelial cells of the oral cavity is often accompanied by an aberrant squamous-cell differentiation, and most of the oral cancers are squamous-cell carcinomas. The pattern of expression of biochemical markers for squamous-cell differentiation of normal oral mucosa epithelial cells and carcinomas and their modulation by retinoids have not been analyzed extensively. Although all of the HNSCC cell lines used in the present study were derived from tumors that had been histologically categorized as squamous-cell carcinomas, they express different levels of type-I TGase. Five of 7 HNSCCs expressed significant levels of type-I TGase activity. The 1483 cells possessed the highest TGase level, whereas cell lines UMSCC-30 and -35 possessed only negligible enzymatic activity. RA and other retinoids decreased the level of type-I TGase in normal rodent and human keratinocytes and tracheobronchial epithelial cells (Jetten and Shirley, 1986; Jetten *et al.*, 1987, 1989*a,b;* Lichti and Yuspa, 1988; Lichti *et al.*, 1985; Parenteau *et al.*, 1986; Rubin and Rice, 1986; Rubin *et al.*, 1989) and suppressed envelope cross-linking (Green and Watt, 1982; Ke *et al.*, 1988; Nagae *et al.*, 1987; Rearick *et al.*, 1988; Yaar *et al.*, 1981). Likewise, RA suppressed type-I TGase in some human SCC cells cultured in the presence of 1.8 mM Ca^{2+} from less than 20% to over 90% (Rubin and Rice, 1986; Rubin *et al.*, 1989; Thacher *et al.*, 1985). In our study RA decreased type-I TGase level in 3 (1483, UMSCC-10A and -22B) of the 5 HNSCC cell lines that expressed significant TGase activity, whereas TGase level in HNSCC 183 and UMSCC-19 was not altered by RA treatment.

RA increased the level of type-II (soluble) TGase, which is not related to squamous-cell differentiation, in mouse keratinocytes (Lichti and Yuspa, 1988), and in human malignant keratinocytes SCC-4 and SCC-12B2 (Rubin and Rice, 1986), while decreasing the level of type-I TGase in the same cells. Type-II TGase was expressed at appreciable levels only in 3 of the 7 HNSCCs used in our studies. The level of this enzyme increased 250% in the 183 and the 1483 cells and decreased by 50% in the UMSCC-19 cells.

CS and the activity of the enzyme cholesterol sulfotransferase, which increase during squamous-cell differentiation, were detected in some of the HNSCCs studied here. CS level was suppressed by RA in 5 of the 6 cell lines that expressed this marker. This decrease could be explained, at least in part, by the observed decrease in the activity of cholesterol sulfotransferase. However, in some cells there is no close correlation between the reduction by RA of cholesterol sulfotransferase activity and CS level. For example, CS level decreased after RA treatment of the 1483 cells by >90%, whereas cholesterol sulfotransferase activity was suppressed by 77%. Conversely, in the UMSCC-22B there was a greater suppression of the enzymatic activity than of the CS level. These differences may be the result of different modulation by RA of the interplay between CS synthesis and its degradation in the individual cell lines.

In normal epidermal and tracheo-bronchial cells the levels of type-I TGase and CS increase concurrently during squamous-cell differentiation, suggesting that their expression is regulated by a joint control mechanism (Rearick and Jetten, 1986; Rearick *et al.*, 1987*a,b*). However, there seems to be no correlation between the expression of these markers in the HNSCC cell lines. For example, the UMSCC-35 cells expressed a very low level of TGase but expressed the highest level of CS, and the 1483 cells expressed the highest type-I TGase level, but the CS level in these cells was not higher than in 3 other cell lines. Further, the extent to which each marker is modulated by RA is distinct for each cell line. Thus, RA treatment of the HNSCC 1483 cells decreased the level of type-I TGase to about 50%, but the CS level was lowered to less than 10% of the control value. Another example is the HNSCC 183 cell line in which RA decreased CS level by more than 60% without altering the type-I TGase level. This observation is reminiscent of the report on uncoupling of the expression of type-I TGase and involucrin in human SCC-9 cells by treatment with certain carcinogens (Rice *et al.*, 1988).

In cultured normal tracheo-bronchial cells, confluence leads to terminal cell division, which is followed by the expression of TGase and CS (Jetten, 1987). RA does not exert any effect on the growth of these cells but suppresses the expression of

the differentiation markers (Jetten, 1987). In contrast, the growth of most of the HNSCCs is suppressed by RA. This growth inhibition does not appear to be related to the level of the differentiation markers or to the effects of RA on their level. Thus, the UMSCC-30 cells, which exhibit very low levels of both TGase type I and CS and, therefore, appear to be a poorly differentiated HNSCC, are inhibited by RA both in monolayer culture and in agarose to the same extent as HNSCC 1483 and UMSCC-10A, which expressed both differentiation markers and appear to be well-differentiated cell lines. Further, the growth of the 183 cells was not inhibited, yet the CS level was decreased by RA. These findings support the contention that in HNSCC cells there may be an uncoupling between the regulation of growth and differentiation, which seem to be coupled in the program of squamous differentiation as delineated in normal keratinizing cells (Jetten, 1987).

Although malignant transformation often results in aberrant expression of differentiation and response to differentiation-inducing stimuli (Parkinson, 1985; Rheinwald and Beckett, 1983; Willey *et al.*, 1985; Willey *et al.*, 1984), some transformed cells and carcinoma cells retain various levels of expression of squamous-cell differentiation markers and responsiveness to retinoids (Fitzgerald *et al.*, 1986; Ke *et al.*, 1988; Reiss *et al.*, 1985; Rubin and Rice, 1986; Thacher, 1989; Thacher and Rice, 1985). Our results demonstrate that this is also the case for most HNSCC cells. Further, we have shown that the growth of the majority of the HNSCC cell lines is inhibited by RA and that the level of one or both squamous differentiation markers is lowered. These results suggest that RA restores some of the normal growth control mechanisms in the malignant HNSCC cells.

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