Cholesterol Sulfate Accumulation in Tumorigenic and Nontumorigenic Rat Esophageal Epithelial Cells: Evidence for Defective Differentiation Control in Tumorigenic Cells

J. I. Rearrick, G. D. Stoner, M. A. George, and A. M. Jetten

Department of Biochemistry, Kirksville College of Osteopathic Medicine, Kirksville, Missouri 63501 [J. I. R.]; Department of Pathology, Medical College of Ohio, Toledo, Ohio 43699 [G. D. S.]; and Cell Biology Group, Laboratory of Pulmonary Pathobiology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 [M. A. G., A. M. J.]

ABSTRACT

In this study the regulation of squamous cell differentiation in several rat esophageal epithelial cell lines is examined. Nontumorigenic RE-149 cells undergo a program of squamous cell differentiation at confluence. This program of differentiation is influenced by the concentration of calcium in the medium and by the presence of retinoic acid. High calcium concentration stimulates terminal cell division, as indicated by a reduction in colony-forming efficiency, and increases the expression of the differentiated phenotype as indicated by an increase in cholesterol sulfate accumulation and cross-linked envelope formation. Retinoic acid inhibits squamous cell differentiation as both cholesterol sulfate accumulation and cross-linked envelope formation are reduced. Two tumorigenic cell lines, RE-B2 and RE-2BT, do not undergo squamous cell differentiation in vitro. High calcium concentration in the medium did not significantly reduce colony-forming efficiency or induce cross-linked envelope formation. High calcium concentration or retinoic acid had only a limited effect on the accumulation of cholesterol sulfate. RE-B2T cells exhibit high levels of cholesterol sulfate and cholesterol sulfotransferase activity. These levels appear no longer controlled by calcium or retinoic acid, indicating that the synthesis of cholesterol sulfate occurs in a constitutive manner. The altered responses of RE-B2 and B2T cells to calcium and retinoic acid suggest that these malignant cells have acquired one or more defects in the control of differentiation.

INTRODUCTION

In certain regions of the world the esophagus is a major site for cancer, and esophageal cancer is a major cause of death (1, 2). Dietary habits and both tobacco and alcohol consumption have been implicated as important risk factors for the development of esophageal cancer (1–5). Our laboratory (G. D. S.) has been involved in the development of in vitro systems to investigate the effects of carcinogens on rat esophageal epithelial cells (6–8). Culture conditions for the serial propagation of normal rat esophageal cells have been developed (9), and cells have been transformed following exposure to the carcinogen, N-nitrobenzylmethylamine (7). Epithelial cell lines have been developed that, following in vitro transplantation into syngeneic rats, produce well-differentiated squamous cell carcinomas (6, 7).

The lining of the esophagus consists of a stratified, squamous cell epithelium. Since the transformation of normal cells is usually associated with alterations in the control of proliferation and differentiation (10), a major objective of studies in in vitro carcinogenesis has been to identify the defects in these controls that lead to a transformed phenotype. In this paper we compare the regulation of squamous cell differentiation in nontumorigenic and tumorigenic esophageal epithelial cells and examine the effects of two modulators of this pathway of differentiation: calcium ions and retinoic acid. Increasing calcium ion concentration in the medium has been shown to stimulate squamous cell differentiation in several in vitro systems (11, 12) including rat esophageal cells (9), whereas retinoids are potent inhibitors of this differentiation process (13–15). In this paper the accumulation of cholesterol sulfate and the expression of steroid sulfotransferase activity were examined during the differentiation process. Our findings indicate that tumorigenic cells acquire defects in the control of differentiation as indicated by altered responses to calcium and retinoic acid.

MATERIALS AND METHODS

Culture Medium. All experiments with RE-149, RE-B2, and RE-2BT were carried out in REM-1 medium. This medium is a modification of PFMR-4 medium (16, 17) in which calcium was reduced from 1.0 to 0.1 mM and contained 5 mg/ml of EGF, 1 mM hydrocortisone (Steroids, Wilton, NH), 1 mM each of ethanolamine and phosphoethanolamine (Calbiochem-Behring, LaJolla, CA), and 5 mg/ml of dialyzed fetal bovine serum protein (9). Gentamicin (Schering Corp., Kenilworth, NJ) was added to the medium at a concentration of 50 µg/ml.

Fetal bovine serum was purchased from Flow Laboratories, McLean, VA (Lot 29101084) and was selected from several commercial lots based upon its ability to stimulate the clonal growth of rat esophageal epithelial cells (9). Fetal bovine serum was dialyzed as described by Kaighn et al. (18), and the protein content of the dialyzed serum was determined by the method of Lowry et al. (19). Colony-forming efficiency, defined as the percentage of plated cells able to form colonies, was determined as described previously (20).

Derivation of Cell Lines. Esophagi were obtained from 4- to 6-week-old F344 rats. Methods used for collection of the esophagi, progressive enzymatic dissociation of the epithelium, and initiation of primary cultures were as described previously (9). Cell line RE-149 is an immortalized cell line derived "spontaneously" from primary cultures of normal rat esophageal cells. Cell line RE-B2 was derived from an outgrowth of epithelial cells from an explant of F344 rat esophagus that had been treated in primary culture with 5 µg per ml of NBMA. The characteristics of this cell line have been described (7). Cell line RE-2BT was derived by enzymatic dissociation of a squamous cell carcinoma produced within 7 mo after s.c. inoculation of RE-B2 cells into the intrascapular region of 1-day-old F344 rats. As indicated in Table 1, RE-B2T cells have a higher colony-forming efficiency in agarose (2.53 versus 0.04%) and are more strongly tumorigenic than RE-B2 cells.

Assay of Cross-Linked Envelopes. For the determination of the spontaneous formation of cross-linked envelopes, medium and cells were collected and centrifuged, and the pellet was resuspended in 20 mM dithiothreitol and 2% sodium dodecyl sulfate. After incubation for 5 min at 95°C, detergent-resistant envelopes were counted as described previously (20). The same procedure was followed to measure the competence to form cross-linked envelopes except that cells were first treated with 25 µg/ml of Ca2+-ionophore Ro 2-2985 (Hoffmann-La Roche, Inc.) in the presence of 2 mM Ca2+. The abbreviations used are: EGF, epidermal growth factor; NBMA, N-nitrobenzylmethylamine.
Assay of Cholesterol Sulfate. Cholesterol sulfate was determined as described previously (22). Briefly, cells were labeled with 20 to 40 μCi/ml of Na[^35]SO₄ (carrier-free; ICN, Irvine, CA) for 24 h, harvested by trypsinization, pelleted by centrifugation, and extracted with 4 ml of chloroform:methanol (2:1). After the addition of 1 ml of 0.1 M KCl, the extracts were vortexed and centrifuged, and the upper aqueous phase was removed. The organic phase was extracted once more with 2 ml of CH₂OH/0.1 M KCl. The resulting organic phase was isolated and taken to dryness, and radioactivity was determined. The lower phase was subjected to thin-layer chromatography using a solvent system composed of CHCl₃:CH₃OH:acetone:glacial acetic acid:H₂O (8:2:4:2:1) and Silica Gel G plates (Analtech, Newark, DE). Steroids were visualized as described previously (22).

Assay for Sulphotransferase Activity. Cells, grown in 60-mm dishes, were collected by trypsinization, pelleted by centrifugation, and resuspended in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.3. The suspension was sonicated 3 times for 15 s using a W-225 sonicator (Ultrasonic Heat Systems). Sulphotransferase activity was measured by determining the incorporation of[^35]Sulfate from 3'-phosphoadenosine-5'-phosphosulfate into cholesterol sulfate as described previously (23).

Assay for Transglutaminase Activity. Transglutaminase activity was determined as described previously (20) by measuring the incorporation of[^3H]putrescine (18.6 Ci/mmol; DuPont, Boston, MA) into casein hydrolysate. Under all conditions tested more than 95% of the total transglutaminase activity was associated with the particulate fraction.

RESULTS

Morphological and Growth Properties of Esophageal Cells. Several characteristics of the cells used in this study are summarized in Table 1. RE-149 is an immortal cell line which exhibits many of the differentiation characteristics of normal rat esophageal cells (6–9). Since normal rat esophageal cells are difficult to obtain in large quantities this cell line was chosen to examine several biochemical parameters of squamous differentiation. The proliferation of RE-149 cells was dependent on the presence of both EGF and dialyzed fetal bovine serum in the medium (9). These cells did not form colonies in soft agar and were nontumorigenic in syngeneic hosts. In contrast, RE-B2 cells, which were derived after NBMA treatment of primary cultures, could grow in the absence of EGF or serum. These cells formed colonies in soft agar and were tumorigenic. The RE-2BT cell line was established from one of the tumors induced by RE-2B cells and exhibited an enhanced ability to form colonies in soft agar as well as increased tumorigenicity. As indicated, all these cell lines are aneuploid.

When RE-149 cells were grown to confluence, a pathway of squamous cell differentiation was induced as indicated by the gradual increase in the formation of cross-linked envelopes. High calcium ion concentration in the medium greatly stimulated this pathway of differentiation. Fig. 1A shows the growth of RE-149 cells grown in REM-1 medium containing either 0.1 mM or 1.0 mM Ca²⁺. High Ca²⁺ concentration caused a slight decrease in the exponential growth rate and a significant reduction in saturation density. A comparison of the morphology of the cells is shown in Fig. 2, A and B. RE-149 cells, grown in low Ca²⁺, formed cross-linked envelopes "spontaneously" only when maintained at confluence for longer than 3 days. High Ca²⁺ concentration stimulated the formation of cross-linked envelopes. A quantitation of the cross-linked envelope formation is shown in Fig. 1B. RE-149 cells were competent to form cross-linked envelopes at all stages of the growth curve; this competence was not influenced by Ca²⁺.

Cholesterol Sulfate Synthesis in Esophageal Cells. Recently, we have established that the synthesis of cholesterol sulfate is a sensitive and convenient marker of squamous cell differentiation (22, 24). To determine whether RE-149 cells synthesize cholesterol sulfate during squamous differentiation, cells grown at confluence in the presence of low and high calcium were incubated with[^35]Sulfate for 24 h, harvested, and extracted...
CHOLESTEROL SULFATE IN ESOPHAGEAL EPITHELIAL CELLS

Fig. 2. Morphology of RE-149, RE-B2, and RE-B2T cells. A, confluent RE-149 cells grown in 0.1 mM Ca\(^{2+}\) (Day 4 of growth); B, confluent RE-149 cells grown in 1.0 mM Ca\(^{2+}\) (Day 4 of growth). Arrowheads indicate cross-linked envelope formation. C, confluent RE-149 cells grown in the presence of 1.0 mM Ca\(^{2+}\) and 2 x 10\(^{-7}\) M retinoic acid; D, confluent RE-B2 cells grown in 0.1 mM Ca\(^{2+}\); E, confluent RE-B2 cells grown in 1.0 mM Ca\(^{2+}\); F, confluent RE-B2T cells grown in 0.1 mM Ca\(^{2+}\).

with chloroform/methanol, and the resulting extract was partitioned against 0.1 M KCl. The resulting organic phase was subjected to thin-layer chromatography. The results (Fig. 3) show that most of the radioactivity comigrated with authentic cholesterol sulfate. In other studies we have characterized the labeled product more rigorously and identified it as cholesterol-3-sulfate (22). No qualitative differences in \(^{35}\)S-labeled lipids were identified between cells grown in low and high calcium.

The production of cholesterol sulfate at different stages of the growth curve is shown in Fig. 1C. Cultures of RE-149 cells grown in low calcium showed an increase in cholesterol sulfate after they reached confluence. The presence of high calcium greatly enhanced the rate of cholesterol sulfate accumulation. Under both low and high calcium conditions the increase in cholesterol sulfate preceded the formation of cross-linked envelopes. Fig. 4 shows the Ca\(^{2+}\) dependency of the increase in
Cholesterol sulfate accumulation. Cholesterol sulfate synthesis increased with increasing Ca\textsuperscript{2+} concentrations and started to level off at concentrations greater than 1.0 mM Ca\textsuperscript{2+}.

To examine the cause of the increase in cholesterol sulfate by calcium, the levels of cholesterol sulfotransferase, the enzyme catalyzing the synthesis of cholesterol sulfate, were determined in RE-149 cells. Confluent cultures grown in high calcium exhibited approximately 7-fold higher levels of cholesterol sulfotransferase than confluent cultures grown in low Ca\textsuperscript{2+} (Table 2). The differences in cholesterol sulfate accumulation appeared to be specific changes, since no significant differences in the incorporation of labeled sulfate into chloroform/methanol-insoluble pellet (CM pellet; Table 3) were found.

Transglutaminase type I has functioned in several systems as an indicator for squamous differentiation (12, 25, 26). However, RE-149 cells exhibited relatively high levels of transglutaminase activity at all stages of the growth curve (not shown) consistent with the competence to form cross-linked envelopes at all stages (Fig. 1B). High Ca\textsuperscript{2+} concentrations increased transglutaminase activity by only 2-fold (Table 3). Under all conditions tested, more than 95% of the transglutaminase activity was associated with the particulate fraction of homogenized RE-149 cells, suggesting that the activity consisted of predominantly type I (epidermal) transglutaminase.

**Action of Retinoic Acid.** Retinoids have been reported to inhibit squamous differentiation in several cell systems (13–15). Treatment of RE-149 cells with retinoic acid inhibited the spontaneous formation of cross-linked envelopes (Fig. 2C; Table 3) and inhibited the accumulation of cholesterol sulfate in the presence of either low or high Ca\textsuperscript{2+} concentrations. A concentration dependency of the inhibition by retinoic acid is shown in Fig. 5. Retinoic acid was active at concentrations as low as 10\textsuperscript{-11} M; the 50% effective concentration was 5 \times 10\textsuperscript{-10} M. Retinoic acid had little influence on the transglutaminase activity of RE-149 cells; as in untreated cells all transglutaminase activity was associated with the particulate fraction. No increase in cytosolic transglutaminase (tissue transglutaminase) activity could be demonstrated.

Cholesterol Sulfate Synthesis in Tumorigenic Cells. The two tumorigenic cell lines RE-2B and RE-2BT did not undergo terminal differentiation either at confluence or in the presence of high calcium. Cultures of these transformed cells reached higher cell densities at confluence than RE-149 cells (Fig. 2, D and F). High calcium concentration in the medium had little effect on the rate of proliferation but did cause a significant (approximately 40%) increase in the saturation density (not shown). In addition, high calcium concentration did not cause a significant reduction in colony-forming efficiency (Table 3). Although both cell lines contained high particulate transglutaminase activity and were competent to produce cross-linked envelopes (about 80 to 90% of cells formed envelopes as in RE-149 cells), no “spontaneous” formation of cross-linked envelopes was induced at confluence or in high calcium (Fig. 2, D and F; Table 3). High Ca\textsuperscript{2+} concentration increased the levels of cholesterol sulfate by 3-fold in RE-2B cells; however, these levels were 10-fold lower than in RE-149 cells. RE-2BT cells exhibited very high levels of cholesterol sulfate at all stages of the growth curve (not shown); these levels were only slightly influenced by either high Ca\textsuperscript{2+} or by the presence of retinoic acid (Table 3). The \textsuperscript{35}S-labeled radioactivity in the organic phase of the extracts from both RE-2B and RE-2BT cells comigrated with cholesterol sulfate when analyzed by thin-layer chromatography (not shown). The high levels of cholesterol sulfate in

---

**Fig. 3.** Thin-layer chromatography of \textsuperscript{35}S sulfate-labeled organic-soluble material from RE-149 cells grown at low (A) or high (B) calcium ion concentrations. The arrows indicate the migration positions of the standards (1, cholesterol sulfate; 2, cholesterol), which were chromatographed in the same lanes as the radioactivity. Only part of the total radioactivity was used for chromatography.

**Fig. 4.** Stimulation of cholesterol \textsuperscript{35}S sulfate synthesis as a function of the calcium concentration. RE-149 cells were plated at 10\textsuperscript{4} cells/60-mm dish, and 2 days later they were changed to the indicated calcium concentration. After 4 more days the synthesis of cholesterol sulfate was determined. Medium was renewed every 24 h. The graph is representative for two independent experiments.

**Table 2 Comparison of the levels of cholesterol sulfotransferase activity in rat esophageal epithelial cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Condition</th>
<th>Cholesterol sulfotransferase (dpm/µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE-149</td>
<td>0.1 mM Ca\textsuperscript{2+}</td>
<td>40 (1)*</td>
</tr>
<tr>
<td></td>
<td>1.2 mM Ca\textsuperscript{2+}</td>
<td>274 (6.9)</td>
</tr>
<tr>
<td>RE-B2</td>
<td>0.1 mM Ca\textsuperscript{2+}</td>
<td>2.5 (1)</td>
</tr>
<tr>
<td></td>
<td>1.2 mM Ca\textsuperscript{2+}</td>
<td>2.0 (0.8)</td>
</tr>
<tr>
<td>RE-B2T</td>
<td>0.1 mM Ca\textsuperscript{2+}</td>
<td>1377</td>
</tr>
</tbody>
</table>

* Numbers between parentheses, fold increase.
CHOLESTEROL SULFATE IN ESOPHAGEAL EPITHELIAL CELLS

Table 3  Effect of calcium and retinoic acid on the accumulation of cholesterol sulfate, transglutaminase activity, cross-linked envelope formation, and colony-forming efficiency in rat esophageal epithelial cells

Cells were grown in the presence of either no additions or 1.0 mM Ca" (final concentration) and/or 2 x 10^-7 M retinoic acid. [35S]Sulfate incorporation into cholesterol sulfate and into the chloroform/methanol-insoluble pellet (CM pellet), transglutaminase activity, cross-linked envelope formation, and colony-forming efficiency were determined during the exponential phase and 3 days after the cell reached confluence. Differences in duplicate values of the cholesterol sulfate determination were 15% or less. The transglutaminase activity is shown as total cellular activity.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Cholesterol [35S]Sulfate (dpm/μg protein)</th>
<th>[35S]Sulfate in CM pellet (dpm/μg protein)</th>
<th>Transglutaminase activity (cpm/h/μg protein)</th>
<th>Spontaneous CE* formation (%)</th>
<th>Colony-forming efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE-149</td>
<td>Exp.</td>
<td>2.7 (1)*</td>
<td>215</td>
<td>65.0 ± 3.3 [96]*</td>
<td>&lt;0.5</td>
<td>30.5 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>Confl.</td>
<td>8.5 (3.1)</td>
<td>205</td>
<td>79.3 ± 5.0 [97]</td>
<td>3.0 ± 0.1</td>
<td>25.9 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>+Ca&quot;</td>
<td>183 (79.6)</td>
<td>187</td>
<td>144.4 ± 7.1 [98]</td>
<td>34.5 ± 0.1</td>
<td>31.1 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>+RA</td>
<td>4.6 (1.7)</td>
<td>206</td>
<td>92.5 ± 4.2 [97]</td>
<td>&lt;0.5</td>
<td>27.3 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>+Ca&quot;/RA</td>
<td>24.8 (9.2)</td>
<td>217</td>
<td>101 ± 3.6 [97]</td>
<td>3.0 ± 1.5</td>
<td>20.8 ± 3.1</td>
</tr>
<tr>
<td>RE-B2</td>
<td>Exp.</td>
<td>5.1 (1)</td>
<td>292</td>
<td>51.8 ± 2.1 [97]</td>
<td>0</td>
<td>49.9 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>Confl.</td>
<td>7.2 (1.4)</td>
<td>339</td>
<td>56.2 ± 2.0 [98]</td>
<td>0</td>
<td>53.0 ± 6.6</td>
</tr>
<tr>
<td></td>
<td>+Ca&quot;</td>
<td>22.6 (4.4)</td>
<td>320</td>
<td>85.0 ± 3.7 [99]</td>
<td>0</td>
<td>47.7 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>+RA</td>
<td>6.1 (1.2)</td>
<td>296</td>
<td>59.3 ± 1.4 [98]</td>
<td>0</td>
<td>51.3 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>+Ca&quot;/RA</td>
<td>11.6 (2.2)</td>
<td>304</td>
<td>83.9 ± 4.2 [95]</td>
<td>0</td>
<td>48.0 ± 2.8</td>
</tr>
<tr>
<td>RE-B2T</td>
<td>Exp.</td>
<td>77 (1)</td>
<td>52</td>
<td>63.0 ± 3.0 [93]</td>
<td>0</td>
<td>50.1 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>Confl.</td>
<td>77 (1)</td>
<td>74</td>
<td>68.1 ± 5.6 [90]</td>
<td>0</td>
<td>51.3 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>+Ca&quot;</td>
<td>121 (1.6)</td>
<td>77</td>
<td>89.7 ± 6.1 [97]</td>
<td>0</td>
<td>48.7 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>+RA</td>
<td>46 (0.6)</td>
<td>72</td>
<td>70.8 ± 4.3 [95]</td>
<td>0</td>
<td>52.6 ± 1.5</td>
</tr>
</tbody>
</table>

* CE, cross-linked envelope; Exp., exponential phase; NA, no additions; Confl., 3 days after cell confluence; RA, retinoic acid.
* Numbers in parentheses, fold increase.
* Mean ± SE.
* Numbers in brackets, percentage of activity associated with the particulate fraction.

Fig. 5. Inhibition of cholesterol sulfate accumulation in RE-149 cells as a function of the retinoic acid concentration. Cells were grown until confluence and then treated with various concentrations of retinoic acid for 4 days. Medium was renewed every day. The graph shown is representative for two independent experiments.

RE-B2T were at least partially related to the relatively high levels of cholesterol sulfotransferase activity (Table 2).

DISCUSSION

The results described in the present report show that tumorigenic rat esophageal epithelial cells exhibit alterations in the control of proliferation and differentiation. The nontumorigenic RE-149 cells undergo a program of squamous cell differentiation when maintained at confluence. They undergo terminal cell division as indicated by a reduction in colony-forming efficiency and start to express a squamous differentiated phenotype as indicated by the production of cholesterol sulfate and the formation of cross-linked envelopes. This program of differentiation is influenced by the concentration of calcium in the medium and by the presence of retinoic acid. High calcium stimulates the expression of the differentiated phenotype as indicated by the increase in cholesterol sulfate accumulation and cross-linked envelope formation. In addition, the growth of the cells was inhibited in high calcium. These observations coincide with, and add significantly to, earlier reports (6, 9) in which we found that the nontumorigenic cells were larger and more polygonal in shape and contained more tonofilaments and junctional complexes when grown in REM-1 medium containing high (1 mM) calcium. In addition, the clonal growth of these cells was inhibited in high calcium (6, 9). A stimulation of squamous cell differentiation by calcium has also been reported for epidermal keratinocytes and tracheobronchial epithelial cells (11, 12, 24, 27-29).

Retinoids have been found to inhibit squamous cell differentiation of various cell systems in vivo as well as in vitro (12-14, 20, 24, 26, 28). Treatment of RE-149 cells with retinoic acid results in an inhibition of the expression of the squamous cell phenotype: both cholesterol sulfate accumulation and cross-linked envelope formation are reduced. Our findings and those of others (11, 12, 14, 26, 28) on the effects of calcium and retinoic acid indicate an antagonism between the two agents. Whether the Ca" ions and retinoic acid affect the expression of the differentiated phenotype via two independent mechanisms or whether they act upon a single target in an opposite direction is an intriguing question that remains unanswered. In any event, it appears likely that calcium and retinoic acid influence the differentiation of epithelial cells derived from different organs in a similar manner.

The tumorigenic cells RE-B2 and B2T do not undergo squamous cell differentiation in in vitro culture when maintained at confluence or in the presence of high calcium ion concentration. No reduction in colony-forming efficiency or induction of cross-linked envelope formation was observed, and high Ca" had only a limited effect on the accumulation of cholesterol sulfate.
RE-2BT cells exhibited high levels of cholesterol sulfate and cholesterol sulfotransferase activity at all stages of growth. These levels were independent of the presence of calcium or retinoic acid. These results suggest that the synthesis of cholesterol sulfate in RE-B2T cells occurs in a constitutive manner and is no longer controlled by calcium or retinoins as is the case for transglutaminase activity. The altered responses of RE-B2 and B2T cells to calcium and retinoic acid, both of which are modulators of squamous cell differentiation in normal cells, suggest that these malignant cells have acquired one or more defects in the control of differentiation, as has been reported for epidermal keratinocytes (30, 31). Possibly, activation of specific oncogene(s) by the carcinogen NBMA might be involved in this process. It is of interest to note in this regard that several 7,12-dimethylbenz(a)anthracene-transformed mouse epidermal keratinocyte cell lines which are not responsive to high Ca2+ contain an activated Ha-ras (32). Experiments with temperature-sensitive mutants in the Ha-ras gene have demonstrated that the ras gene can induce resistance to terminal differentiation (33). These results suggest a correlation between the expression of Ha-ras and Ca2+ responsiveness. How the activated Ha-ras alters the responsiveness to Ca2+ has to be determined.

As reported previously for tracheobronchial epithelial cells (22-24), squamous differentiation of rat esophageal cells is associated with increased accumulation of cholesterol sulfate. This increase in cholesterol sulfate appears to be largely related to an increase in cholesterol sulfotransferase activity (Table 2; Ref. 23) and precedes the formation of cross-linked envelopes. These findings suggest that increased cholesterol sulfate accumulation and sulfotransferase activity are early biochemical changes during squamous differentiation of epithelial cells from a number of different tissues. However, the results with RE-2BT cells indicate that increased cholesterol sulfate levels do not necessarily mean that cells express a squamous differentiated phenotype. As mentioned above the expression of high cholesterol sulfate levels as the expression of transglutaminase type I activity in transformed cells can be uncoupled from terminal differentiation. The function of cholesterol sulfate accumulation during squamous cell differentiation is not known. However, cholesterol sulfate is present in significant quantities in the stratum corneum of the skin and may play a role in the regulation of lipid metabolism (34-36). Alternatively, cholesterol sulfate might function as a receptor for specific proteins involved in the cornification process or affect the activity of certain proteins in the membrane. The correlation between cholesterol sulfate accumulation and cholesterol sulfotransferase activity among the three RE-cell lines was not perfect. One possible explanation is that these cell lines also differ in the levels of sulfatase activity that hydrolyses cholesterol sulfate.

In summary, using colony-forming efficiency, the production of cholesterol sulfate, and the formation of cross-linked envelopes as markers for terminal squamous cell differentiation, our results show that the tumorigenic cell lines RE-B2 and B2T have acquired one or more defects in the control of terminal differentiation. The esophageal cell lines described in this paper appear to provide a good model system to study the process of differentiation and carcinogenesis in the esophagus. Studies are underway to determine whether activation of specific oncogenes is involved in the altered control of differentiation in these cells and what its relation might be to the loss of Ca2+ responsiveness.

ACKNOWLEDGMENTS
The technical assistance of Anthony Gallart is most appreciated.

REFERENCES


Cholesterol Sulfate Accumulation in Tumorigenic and Nontumorigenic Rat Esophageal Epithelial Cells: Evidence for Defective Differentiation Control in Tumorigenic Cells

J. I. Rearick, G. D. Stoner, M. A. George, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/18/5289

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.