Increase in Cholesterol Sulfotransferase Activity during in Vitro Squamous Differentiation of Rabbit Tracheal Epithelial Cells and Its Inhibition by Retinoic Acid*

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It has previously been demonstrated that rabbit tracheal epithelial cells in primary culture undergo terminal differentiation at confluence to yield cornified cells much in analogy to epidermal keratinocytes and that one biochemical marker of this process seems to be the accumulation of cholesterol sulfate by the cells. The current work addresses the possible causes of this accumulation. Our studies show that the stimulation of cholesterol sulfate is paralleled by an increased activity of the biosynthetic enzyme cholesterol sulfotransferase. Squamous differentiated cells exhibited 20- to 30-fold higher levels of this enzyme activity than that in undifferentiated cells. As with other markers of squamous cell differentiation, the increase in cholesterol sulfotransferase can be prevented by the inclusion of retinoids in the cell culture medium. Inhibition of sulfotransferase levels can be observed at concentration of retinoic acid as low as 10⁻¹¹ M. The enzyme activity is optimal at pH 7 in buffers containing 0.2 M NaCl and 0.01% Triton X-100. Apparent Michaelis constants for the substrates 3'-phosphoadenosine-5'-phosphosulfate and cholesterol are 1 µM and 0.6 mM, respectively. Our results indicate that the increase in cholesterol sulfotransferase is the proximate cause for the accumulation of cholesterol sulfate in rabbit tracheal epithelial cells during squamous cell differentiation.

Under conditions of vitamin A-deprivation or toxic or mechanical injury, the normally mucociliary epithelial lining of the mammalian respiratory tract undergoes squamous metaplasia (1-3). The changes which occur appear to be related to the expression of an alternate differentiation pathway. To study the regulation of differentiation of tracheobronchial epithelial cells, an in vitro model was developed using rabbit tracheal epithelial (RbTE) cells (4). These studies have indicated that squamous cell differentiation of tracheobronchial epithelial cells is a multistep process (5-8). In the first stage cells become committed to terminal cell division; this is followed by the expression of the squamous cell phenotype. The latter is characterized by morphological changes, formation of cross-linked envelopes and alterations in biochemical parameters such as keratin expression (9, 10), type 1 transglutaminase activity (11), and expression of two squamous cell-specific RNAs.² Squamous cell differentiation and expression of the biochemical markers associated with it occur after cells reach the confluence phase of the growth curve and can be induced at low density by the addition of transforming growth factor β to the medium (12). Inclusion of retinoids in the medium inhibits squamous cell differentiation (9-12). We have observed that increases in [³⁵S]sulfate incorporation into cholesterol sulfate correlate well with squamous cell differentiation in vitro, in particular with the formation of cross-linked envelopes (13, 14). Confluency or addition of transforming growth factor β leads to enhanced levels of cholesterol sulfate (12, 13). This accumulation of cholesterol sulfate is inhibited by retinoids. They apparently coordinate regulation of cholesterol sulfate synthesis with other well-established markers of squamous cell differentiation leads us to propose that it be considered a new marker of this process.

Formally, one could envision several possible proximal causes of the increase in [³⁵S]sulfate incorporation into cholesterol sulfate. These include: (i) decreased activity of the degradative enzyme steroid sulfatase, (ii) increased activity of the biosynthetic enzyme cholesterol sulfotransferase, or increased amounts of the substrates of the latter enzyme, either (iii) cholesterol or (iv) PAPS. An increase in PAPS has previously been ruled out since no overall increase in other sulfation reactions in the RbTE cells was observed (13). In the current work we address the other three possibilities mentioned above and conclude that increases in cholesterol sulfotransferase activity which occur during in vitro squamous cell differentiation of RbTE cells appear to be the main cause for the increased levels of cholesterol sulfate.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Metabolic Radiolabeling.** Rabbit tracheal epithelial cells were isolated by protease digestion, plated at 5 × 10⁶/dish on fibronectin/albunin/Vitrogen-coated 60-mm dishes and cultured in Ham’s F-12 medium as previously described (4, 11). Cells were metabolically radiolabeled by incubating for 22 h with carrier-free Na₂[³⁵S]SO₄ (ICN, Irvine, CA) at 10 µCi/ml of medium. Cell pellets obtained after trypsinization were extracted with organic solvents and the incorporation of radioactivity into cholesterol sulfate determined as previously described (13).

**Assay for Cholesterol Sulfotransferase Activity.** Cells were harvested by trypsinization, pelleted by centrifugation, and the resulting pellet was resuspended in buffer containing 0.6 M HEPES, pH 7.3. The suspension was sonicated 3 times for 15 s each using a W-225

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The abbreviations used are: RbTE cells, rabbit tracheal epithelial cells; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; PIPEs, piperaclie-N, N'-bis(2-ethanesulfonic acid).

sonicator (Ultrasonic Heat Systems) equipped with a microprobe. In some experiments, a freeze-thaw procedure was substituted for sonication. Suspensions were alternatively frozen in a dry ice-ethanol bath and thawed in a 40 °C water bath 3 times. After centrifugation (15,000 × g, 15 min, 4 °C), the supernatant was removed. Standard assay mixtures contained 50 mM HEPES, pH 7.3, 0.01% Triton X-100, 200 mM NaCl, 2.6 mM cholesterol, 1.8 μCi of PAPS (104 dpm), and 5% amounts of enzyme from the supernatant described above in a final volume of 100 μl. Unless otherwise indicated, incubations were performed at 37 °C for 60 min. The reactions were terminated by the addition of 4 ml of chloroform/methanol (2:1, v/v), followed by the addition of 8 ml of 0.1 M KCl. After vortexing, centrifugation, and removal of the aqueous upper phase, the lower phase was transferred to a scintillation vial, taken to dryness under a nitrogen stream, and radioactivity was determined after the addition of 0.4 ml of H2O and 4 ml of Hydrofluor (National Diagnostics Inc).

Product Characterization—To generate large amounts of radiolabeled enzyme product, a reaction mixture 10-fold larger than that described above was prepared containing 2.2 mg of enzyme protein and with the following changes: the exogenously added acceptor substrate was 0.1 mCi (23.7 Ci/mmol) of [7-3H]cholesterol, while the PAPS (1.8 μM) was 2.6 × 106 dpm. Incubation for 2 h at 37 °C resulted in a [35S]sulfate incorporation of 2.0 × 106 dpm in the lower organic phase after chloroform/methanol (2:1) extraction and partitioning. Radioactive material partitioning in the lower phase was subjected to anion exchange chromatography, followed by thin layer chromatography.

Steroid Sulfatase Assay—Enzyme solutions were prepared in the same way as for cholesterol sulfotransferase assays, except that both supernatant and pellet were assayed for enzyme activity. The reaction mixture contained 50 mM HEPES, pH 7.3, 0.5 mM dithiothreitol, 0.02% Triton X-100, 5 × 106 dpm of cholesterol [35S]sulfate and amounts of enzyme protein which varied up to 380 μg. Unless otherwise indicated, incubations were performed at 37 °C for 60 min. The reactions were terminated by the addition of 4 ml of chloroform/methanol (2:1), followed by the addition of 1 ml of 0.1 M KCl. After vortexing and centrifugation, the upper aqueous phase was removed and the chloroform/methanol (2:1) extraction and partitioning resulted in a [35S]sulfate incorporation of 2.0 × 106 dpm in the lower organic phase after chloroform/methanol (2:1) extraction and partitioning. Radioactive material partitioning in the lower phase was subjected to anion exchange chromatography, followed by thin layer chromatography.

Chromatography—Thin layer separations were accomplished with the following solvent systems: I, methyl ethyl ketone/benzene/ethanol/H2O (3:3:3:1); II, CHCl3/CH3OH/acetic/glacial acetic acid/H2O (8:2:4:2:1); III, tetrahydrofuran, methyl, CH3OH, 4 M (60:30:10:4). Plates precasted with silica gel (Analtech) were used. Sterols were stained using acidic iron trichloride (18).

Ion exchange chromatography of organic solvent extracts was performed on a column (5.0 × 0.85 cm) of DEAE-Sephadex A-25 equilibrated in chloroform/methanol (1:1). The sample was applied in chloroform/methanol (1:1) and the column was washed with the same solvent. The column was then eluted sequentially with 10, 20, 30, and 50 mM ammonium acetate in chloroform/methanol (1:1). Pool fractions containing ammonium acetate were desalted by partitioning as described above. Such desalting resulted in >90% recovery of radioactivity in the lower phase.

Cellular Half-life of Cholesterol 35S/Sulfate—Cells were incubated with Na2[35S]SO4 for 24 h beginning either on day 6 after plating (proliferative cells) or on day 11 (confluent and retinoic acid-treated cells). Proliferative and retinoic acid-treated cells were incubated at 50 μCi/ml, while confluent cells were incubated at 10 μCi/ml. After 24 h, the radioactive medium was removed, the dishes were rinsed twice with Dulbecco's phosphate-buffered saline, and fresh medium was added. Cells were harvested daily for 4 or 5 days and the amount of radioactivity remaining in cholesterol sulfate was determined as described above. Determinations were performed in duplicate on the cells from individual dishes, except with the proliferative cells, where the cells from four dishes were pooled to yield each duplicate. Initial levels of incorporation into cholesterol [35S]sulfate were 8.2 × 105 and 184 dpm/μg protein for proliferative, retinoic acid-treated, and confluent cells, respectively.

Determination of pH Optimum—To measure the pH optimum of the cholesterol sulfotransferase the assay was carried out in the following pH-buffer systems: from pH 5.1 to pH 7.37, 0.05 M HEPES (from pH 6.6 to 8.54), 0.05 M Tris (from pH 7.5 to 9.0), and 0.025 M PIPES (from pH 6.45 to 7.94).

Cholesterol Determination—Total cholesterol was determined by a microscale modification of the ferric chloride procedure described by Kates (16) ([1.0 ml final volume). Cholesterol ester was determined similarly, after removal of free cholesterol from a hexane/diethyl ether (90:10, v/v) solution of total lipids by adsorption on Florisil.

RESULTS

Crude sonicates of confluent RbTE cells are able to transfer 35SO4 from PAPS to lipophilic acceptors as detected by the appearance of radioactivity in the lower phase of a partitioned organic solvent extract of reaction mixtures. The putative cholesterol sulfotransferase activity is linear with respect to time of incubation up to 90 min and with respect to cellular protein added up to at least 240 μg (results not shown). As indicated in Table I, the activity in crude solution is only partly dependent on exogenously added cholesterol, with reaction mixtures containing no added cholesterol giving activities around 25–50% of those obtained at saturating concentrations. The use of freeze-thawing rather than sonication had no effect on the apparent activity in the absence of exogenous cholesterol but increased the activity at 1 mg/ml cholesterol about 2-fold. Centrifugation of the extract from either sonicated or freeze-thawed cells at either 13,000 × g for 15 min (Table I) or 100,000 × g for 1 h (results not shown) was ineffective in pelleting the endogenous acceptor substrate. At forces of 470,000 × g for 4.5 h, however, the endogenous acceptor is pelleted while the enzyme activity remains in the supernatant solution. A portion of the endogenous acceptor is easily resuspended, since careful aspiration of the final 0.5 ml of enzyme solution from the bottom of the ultracentrifuge tube resulted in a regeneration of the same ratio of activities with and without added cholesterol as the starting material before centrifugation (Table I). Because of the time required and the relatively small number of samples which can be processed simultaneously, the 470,000 × g centrifugation step was used only when necessary to yield unambiguous results.

To characterize the product formed with the 470,000 × g supernatant solution as an enzyme source and using [3H]cholesterol and PAPS as substrates (see “Experimental Procedures”), the radioactive material partitioning in the lower phase was subjected to anion exchange chromatography on DEAE-Sephadex. The results (Fig. 1) indicate that a doubly labeled peak containing all the sulfate radioactivity can be eluted from the column by 20 mM ammonium acetate. When this partially purified labeled material was analyzed by thin layer chromatography (Fig. 2), the [35S]sulfate and tritium radioactivity co-migrated as a single species coincident with authentic cholesterol sulfate chromatographed in the same solvent system.
under "Experimental Procedures." The doubly labeled material contained in the organic phase was applied to a column (0.85 cm) of DEAE-Sephadex equilibrated in CHCl₃/CH₃OH (1:1). After washing with 15 ml of the equilibration solvent, the column was eluted with the indicated concentrations (in millimolar, arrows) of ammonium acetate in CHCl₃/CH₃OH (1:1). The symbols indicate tritium (closed circles) and [³⁵S] open circles).

![Thin layer chromatography of the cholesterol sulfotransferase reaction product](image)

**Fig. 2.** Thin layer chromatography of the cholesterol sulfotransferase reaction product. Doubly labeled material eluting from DEAE-Sephadex with 20 mM ammonium acetate (Fig. 1) was subjected to thin layer chromatography in systems I (panel A) and II (panel B). The symbols are the same as described in the legend to Fig. 1, while the arrows indicate the migration positions of the internal standards, 1, cholesterol sulfate and 2, cholesterol, detected by reaction with acidic iron trichloride (16).

Lane. Similar results (not shown) were generated when the enzyme source used to synthesize the doubly labeled product was a 13,000 × g supernatant solution which contained the endogenous acceptor. This result suggests that the endogenous acceptor is probably cholesterol since presumably most of the transferred sulfate radioactivity in the latter experiment was present on endogenous acceptor rather than on [³H] cholesterol and only a single [³⁵S]sulfated species was obtained.

Varying the components of the reaction mixture to optimize conditions lead to several interesting observations. The pH dependence of the reaction gave a broad optimum centered on pH 7.0 with about 50% maximal activity at either pH 6.0 or 8.0 (not shown). An overlapping set of three buffer salts (MES, PIPES, and HEPES) gave rise to a smooth curve indicating that they support enzymatic activity equally well. Tris buffer on the other hand was inhibitory at all pH values tested, inhibiting 75% at pH 7.5 relative to PIPES and HEPES. The optimum Triton X-100 concentration in the assay was determined to be between 0.005 and 0.01% (Fig. 3, upper), which stimulated activity 7-fold over either no detergent or 0.1%. In a separate experiment (not shown), it was determined that enzyme activity continued to decline monotonically with increasing Triton X-100 concentrations up to 1.5%, the highest concentration tested. Since ion exchange chromatography was an anticipated purification step for the enzyme, the effect of NaCl concentration on enzyme activity was tested. As shown in Fig. 3 (lower), optimal activity was obtained between 150 and 200 mM NaCl which stimulated activity 4- to 5-fold over activity in the absence of NaCl. Higher NaCl concentrations were inhibitory. Preliminary results (not shown) suggest that no complex relationship exists between detergent concentration and NaCl concentration since similar NaCl concentration dependencies have been obtained at different Triton X-100 concentrations.

The dependence of the enzyme reaction upon substrates is shown in the form of double reciprocal plots in Fig. 4. The results indicate that the enzyme has apparent Kₘ values of 1 μM and 0.6 mM for PAPS and cholesterol, respectively, and an apparent Vₘₙₐₓ in each case of 240 pmol/h/mg protein.

Having developed an assay system to measure cholesterol sulfotransferase activity in RbTE cells, we next set about to measure enzyme activity during in vitro squamous differentiation. Cells were harvested representing three growth conditions: (i) proliferative cells which have not yet undergone differentiation, (ii) cells which have been at confluency for several days and have begun to express differentiated characteristics, and (iii) cells at confluency which have been prevented from differentiating by the inclusion of retinoic acid in the culture medium. Table II summarizes the results obtained in measuring the incorporation of [³⁵S]sulfate into cholesterol sulfate by the intact cells and in quantitating the cholesterol sulfotransferase activity present in cell extracts. As previously observed (13), [³⁵S]sulfate incorporation into cholesterol sulfate in confluent cells was enhanced about 50-fold over that in proliferative cells and this increase was prevented by retinoic acid treatment. Likewise, cholesterol sulfotransferase also dramatically increased (at least 20- to 30-fold) in the differentiated confluent cells compared to
either the proliferative or retinoic acid-treated cells. The difference in enzyme activity between retinoic acid-treated and proliferative cells, while marked in this experiment, was not consistently observed, and indeed activities from retinoic acid-treated cells were usually lower than that shown in Table II. Mixing experiments (not shown) utilizing varying proportions of extracts from either proliferative or retinoic acid-treated cells in combination with extracts from differentiated cells yielded results that were strictly additive, indicating that no inhibitor of cholesterol sulfotransferase was present in the undifferentiated cells. Retinoic acid included in the in vitro assay mixture at concentrations ranging from 10^{-11} to 10^{-6} M did not result in any inhibition of cholesterol sulfotransferase activity, demonstrating that retinoids do not directly affect the enzyme (results not shown).

In contrast to the results obtained with cholesterol sulfotransferase, steroid sulfatase activity was not detectable in cells under any of the growth conditions and did not change in a differentiation-related manner (results not shown). As an independent measure of whether cholesterol [35S]sulfate accumulation by the intact cells (13). The enzyme was 35 μl of a 470,000 × g supernatant which was capable of transferring 54,000 dpm/h in a standard reaction mixture including 2.6 mM added cholesterol. None of the 470,000 × g pellets themselves contained appreciable enzyme activity.

![Image](image.png)

**FIG. 4.** Double reciprocal plots of cholesterol sulfotransferase activity versus substrate concentration. Upper, PAPS was varied as the cholesterol concentration was held constant at 2.6 mM. Lower, cholesterol was varied as the PAPS concentration was held constant at 1.8 μM. In each case the reaction mixtures contained 205 μg of enzyme protein from a 470,000 × g supernatant solution.

The availability of acceptor substrate might simply increase the difference in the amount of product formed. As shown in Table II, the in vitro endogenous acceptor does not change significantly among the conditions in a differentiation-related manner, as measured by its ability to serve as an acceptor substrate in the standard reaction mixture.

With evidence in hand that changes in cholesterol sulfotransferase activity are responsible for the differentiation-dependent changes which occur in RbTE cell cholesterol sulfation, a complete time course of the phenomenon was determined. As indicated in Fig. 5, the accumulation of cholesterol [35S]sulfate by the cells begins about 2 days after confluency is reached. As previously noted, this is the same time that other differentiated characteristics begin to appear (4-8). At the same time, cholesterol sulfotransferase activity also increases until a maximum is reached about 6 days after confluency. The subsequent decline in enzyme activity may correlate with cross-linked envelope formation, since in the late stages of cornification, cell digest their intracellular contents during the process of cell death (17, 18).

The complete concentration-dependence of the retinoic acid effect upon cholesterol sulfotransferase activity is shown in Fig. 6. The results indicate that the half-maximal effect of retinoic acid in inhibiting cholesterol sulfotransferase induction occurs at 5 × 10^{-10} M which is consistent with the comparable value (5 × 10^{-10}) obtained for the inhibition of cholesterol [35S]sulfate accumulation by the intact cells (13).

**DISCUSSION**

The results presented show that the activity of the enzyme cholesterol sulfotransferase increases upon squamous cell differentiation of RbTE cells in the culture and that this increase can be prevented by the inclusion of retinoids in the culture medium. Thus, cholesterol sulfotransferase is likely to be a member of a spectrum of proteins, including type I transglu-
ichthyosis, results from a deficiency of the enzyme steroid sulfatase (20, 21) accompanied by an increase in cholesterol sulfate in the stratum corneum (22), it became apparent that levels of cholesterol sulfation are subject to regulatory controls, at least in skin, and that derangements in this regulation could have important pathological consequences. In an effort to complement studies with the sulfatase and extend the possible regulation of cholesterol sulfate levels to include the biosynthetic enzyme, Epstein and co-workers (23) have partially characterized the cholesterol sulfotransferase from mouse epidermis. Their results contain both similarities and interesting differences from the current work.

First, both the epidermal and the RbTE enzymes remain in the supernatant solution after centrifugation at 100,000 × g and act on endogenous acceptor substrates which are also soluble by this criterion. Epidermal cells seem to contain sufficient endogenous acceptor that addition of exogenous cholesterol results in no enhancement of apparent activity. The application of potential purification steps resulted in the separation of cholesterol sulfotransferase from the endogenous acceptor by means of adsorption chromatography using a column of Cibacron Blue-agarose. Use of a similar technique with the RbTE cholesterol sulfotransferase failed even though the enzyme bound to the column and was eluted by NaCl as described for the epidermal enzyme (results not shown). Other separation techniques which failed to remove the endogenous acceptor from the RbTE enzyme included gel filtration, anion exchange chromatography, and adsorption to polystyrene beads.

Other significant differences between the epidermal and RbTE enzymes include their activities in the presence of various buffer components, including Triton X-100, Tris buffer, and NaCl. While detailed Triton X-100 and pH optima for the epidermal enzyme were not presented, it is clear that if the RbTE enzyme were assayed under similar conditions, severe inhibition of activity would result. If these parameters for the epidermal enzyme have indeed been optimized then differences between the two enzymes exist. Differences certainly are apparent for the dependence of enzyme activity on NaCl concentration since NaCl inhibits the epidermal enzyme at all concentrations tested (cf. Fig. 8). These differences illustrate the benefits of optimizing previously established procedures for use in a different tissue for the study of enzymology per se. They also sound a note of warning concerning the enzyme source to be used in generating probes (i.e. enzyme for use as antigen) for use in answering questions about the regulation of cholesterol sulfotransferase activity at the molecular level.

Having demonstrated that cholesterol sulfotransferase activity increases during in vitro differentiation of RbTE cells, the next logical question to ask is whether the regulation of this phenomenon occurs at the level of transcription, translation, or post-translational processing. Such studies require the purification of the enzyme and ultimately the isolation of mRNA coding for the protein, either by use of monospecific antisera to immunoprecipitate polysomes engaged in translation or by sequence analysis of the protein followed by construction of an appropriate unique oligonucleotide probe to identify cholesterol sulfotransferase mRNA. This task may be made somewhat easier by the availability of cDNA clones which correspond to mRNA species which are expressed in a differentiation-related manner by RbTE cells. Use of two of these clones to investigate questions concerning the regulation of their expression has led to the conclusion that while they seem to be transcribed at comparable levels by both differ-

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**FIG. 5.** Time course of appearance of cholesterol sulfotransferase activity in RbTE cells. Cells numbers (closed circles) were determined in triplicate by hemacytometer after trypsinization. Bars indicate standard deviations. Cells for enzyme assays were harvested by trypsinization and sonicated to give crude homogenates used as the enzyme source. The enzyme activity (open circles) was measured as the amount of radioactivity transferred from the donor substrate, PAP35S, into the lower organic phase. Incubations with Na235SO4 (10 μCl/ml) were terminated at the indicated times and incorporation of radioactivity into the lower phase determined (open squares).

**FIG. 6.** Retinoic acid concentration dependence of the inhibition of the appearance of cholesterol sulfotransferase activity. Retinoic acid was added to RbTE cells at the indicated concentrations beginning on day 5 after plating and at each medium change thereafter. On day 13, cells were harvested and homogenized by sonication.

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entiated and undifferentiated cells, the mRNAs produced are much more stable in differentiated cells.

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