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L A Miller, … , Y H Zhao, R Wu

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Research Article

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Inhibition of TGF-α Gene Expression by Vitamin A in Airway Epithelium

Lisa A. Miller, Yu Hua Zhao, and Reen Wu

California Regional Primate Research Center and Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, University of California at Davis, Davis, California 95616

Abstract

The autocrine/paracrine growth mechanism has been implicated in the regulation of bronchial epithelial cell proliferation. By inhibiting the expression of the transforming growth factor-α (TGF-α) gene product, vitamin A is able to suppress the proliferation of tracheobronchial epithelial cells in culture. Similar repressions in TGF-α mRNA levels by retinol were observed in airway explant cultures and in a cell line immortalized from normal human bronchial epithelial cells. Both the nuclear run-on transcriptional assay and the transfection study with the chimeric construct of the TGF-α promoter and chloramphenicol acetyltransferase reporter gene partly suggest a transcriptional downregulation mechanism of TGF-α gene expression by the retinol treatment; however, this inhibition at the transcriptional level cannot account for the total inhibition at the mRNA level. These results suggest that a downregulation of the expression of the TGF-α gene at the transcriptional and post-transcriptional levels by vitamin A may precede the essential event associated with the homeostasis of normal conducting airway epithelium. (*J. Clin. Invest*. 1996. 97:1429–1435.) Key words: growth factor • transcriptional regulation • retinoids • gene expression • autocrine/paracrine

Introduction

The profound effects of vitamin A on the growth and differentiation of epithelia are well recognized. In the lining cells of the respiratory tract, vitamin A is required for the expression of mucociliary differentiation—an essential function for pulmonary defense (1). A vitamin A deficiency results in the phenomenon of squamous cell metaplasia, during which the normal pseudostratified respiratory epithelium thickens and undergoes keratinization. This process has been demonstrated both in vivo and in vitro (1–5). Exposure to toxic inhalants, chronic inflammation, and mechanical stress can also produce similar lesions within the tracheobronchial mucosa (6–8). The pathological association of squamous metaplastic foci with bronchogenic carcinoma, as well as epidemiological evidence correlating vitamin A deficiency with increased lung cancer risk, has prompted considerable interest in elucidating the mechanisms for this alternate pathway of differentiation (9–11). Despite extensive investigation, the origin of metaplastic cells and the induction and physiological consequences of squamous cell differentiation on airway epithelium are still uncertain.

It has been suggested that vitamin A serves as a homeostatic regulator for airway epithelium by mediating the expression of both positive and negative growth factors (12). A deficiency of vitamin A may result in a temporary imbalance of growth factors, leading to a basal cell hyperplasia that typically precedes squamification of epithelium. This potential link between growth abnormality and vitamin A deficiency in airway epithelium led us to examine the effects of vitamin A on growth factor secretion by airway epithelium. Based on data obtained by in vitro studies, it is clear that the proliferation of respiratory epithelium is dependent on a number of growth factors such as the epidermal growth factor (EGF) and insulin (13, 14). The endogenous expression of EGF, TGF-α, and EGF receptors in normal adult and fetal lung has been demonstrated in several species, suggesting that the respiratory epithelium is capable of autocrine/paracrine growth control (15–20). The co-expression of EGF and TGF-α within the lung is enigmatic since both proteins produce mitogenic effects through the same tyrosine kinase receptor, EGF receptor (21). However, there is evidence to support the notion that differential responses are mediated at the receptor level, which suggests distinct functional roles for EGF and TGF-α within the lung (22).

We have previously demonstrated that both human and non-human primate TBE cells are able to secrete an EGF-like growth factor in vitro and this secretion is inhibited by retinol (23). The secretion of this EGF-like growth factor can reduce the cellular growth requirement for EGF and also downregulate EGF receptor levels. The possibility that the EGF-like growth factor produced by primary TBE cell cultures is in fact TGF-α was supported by the presence of elevated TGF-α–specific antigen levels under conditions of vitamin A deficiency. Furthermore, blocking TGF-α from binding to its receptor with a neutralizing antibody reduced the DNA synthesis in vitamin A–deficient TBE cell cultures (23). Cumulatively, these results suggest that in TBE cells, vitamin A plays an important role in regulating an autocrine/paracrine mechanism of growth mediated by an EGF-like mitogen. Our preliminary observations, which associate vitamin A deficiency with enhanced TGF-α secretion, led us to investigate directly the regulation of TGF-α by vitamin A in respiratory epithelium at the nucleic acid level.

Methods

Sources of tissues, cells, and culture conditions. Human tracheobronchial tissues were obtained from organ donor patients or from autopsy through the University of California, Davis, Medical Center (Sacramento, CA), or from the International Institute for Advance Medicine, Inc. (Exton, PA). Rhesus monkey tracheobronchial tissues

Address correspondence to Dr. Reen Wu, California Regional Primate Research Center, University of California at Davis, Davis, California 95616. Phone: 916-752-2648; FAX: 916-752-2880; E-mail: rwu@ucdavis.edu. L.A. Miller's current address is Laboratory of Immunology and Vascular Biology, Department of Pathology, Stanford University Medical Center, Stanford, CA 94305.

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were obtained from the California Primate Research Center of University of California at Davis. Procedures for the tracheobronchial epithelial (TBE) cell isolation and the culture conditions were carried out as previously described (14, 24). Unless it is specified, vitamin A (retinol, 0.1 μM) was initially not added to the culture medium. For organ culture, 1- to 2-mm thick tracheobronchial slices were maintained in the same culture condition on top of a Whatman's paper (No. 3) and the tissues were covered by a very thin layer (~ 1 mm) of serum-free culture medium. The dishes were incubated in a humidified chamber containing 50% O_2, 45% N_2, and 5% CO_2. The medium was changed once every other day. At the end of incubation, tissue slices were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned (4–6 μm thickness) for in situ hybridization (25).

RNA extraction, Northern blot hybridization, and RNase protection assays. Total RNA was isolated by the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method described previously (26). Northern blot hybridization was carried out as previously described (27). Based on the reported human TGF-α cDNA sequence (28), a 350 bp of monkey TGF-α cDNA was generated by the polymerase chain reaction (PCR) method using two primers: TA1 = 5′CTGTTCGCTCTGGGTATTGT3′ and TA2 = 5′ACCTGGCAGCAGTGTATCAG3′. This PCR product was subcloned into PGEM4Z (Promega, Madison, WI), namely pMTA, and the DNA sequence of the cDNA insert was verified by the dideoxynucleotide chain termination DNA sequencing method (29). Both sense and anti-sense cRNA labeled by ^{32}P-α-UTP were produced from this clone and used in an RNase protection assay (RPA).[1] A commercial kit (Ambion Inc., Austin, TX) for RPA was used in this study and the assay was carried out as suggested by the manufacturer's protocol.

RT-PCR methods. The levels of TGF-α message in cultured cells were quantified by Reverse transcriptase-PCR (RT-PCR) methods. The routine RT-PCR was performed as suggested by Kawasaki (30) for 25 cycles with the following conditions: 95°C for 1 min, 55°C for 1 min, and 72°C for 30 s. The PCR products were electrophoretically separated in 1% agarose gel and transferred to Nytran membranes. Southern hybridization with a ^{32}P-TGF-α cDNA probe was performed by the method of Southern (31). The quantitative RT-PCR method was carried out as described by Riedy et al., (32). The mutant TGF-α template (266 bp) was prepared by synthesizing two oligonucleotide primers internal to TA1 and TA2, which are as follows: primer TA3 = 5′GGAATTCGTGTGGGAATCTGGGCAGTC3′ and primer TA4 = 5′GGAATTCGTGAGCATGCGGACCTCCTG3′ (both TA3 and TA4 have EcoR1 restriction sites incorporated). The wild-type monkey TGF-α 350-bp PCR product was used in amplifications with either TA1/TA3 or TA2/TA4 primer pairs to produce 135- and 140-bp PCR products, respectively. The 135- and 140-bp PCR products were ligated together, re-amplified with TA1/TA2, and the resulting 266-bp PCR product was subcloned into PGEM4Z (Promega). Mutant TGF-α cRNA was prepared by in vitro transcription using a commercially available kit (Promega) and quantified by optical density readings. For quantitative RT-PCR, a known quantity of mutant cRNA was included with total RNA. Using TA1 and TA2 primers, the PCR products contained both wild-type form and mutant fragment at 350 and 266 bp, respectively.

Human β-actin specific primers were obtained from a commercial source, which will produce a 650-bp RT-PCR product (Clontech Laboratories, Inc., Palo Alto, CA).

Nuclear run-on assay. Nuclei isolation and in vitro transcriptional reactions were performed as described previously (27). Briefly, more than 10 million nuclei were isolated from retinol-treated and -untreated primary human TBE cultures. These nuclei were incubated in a nuclear run-on reaction solution which contained ^{32}P-a-UTP for incorporation labeling. Run-on RNA were isolated through a single-step acid guanidinium thiocyanate-phenol-chloroform extraction method described previously (26). Radioactive nuclear RNA (5–10 million counts each preparation) were hybridized with DNA slot blot and processed as described before (27).

In situ hybridization. Both ^{35}S-labeled sense and antisense TGF-α cRNAs were generated from a monkey TGF-α cDNA clone, pMTA, using the cRNA generating kit from Ambion Inc. (Austin, TX). Culture dishes and paraffin section slides were hybridized with these cRNA probes. Pretreatment of slides and conditions for hybridization and washing were performed as described by Angerer and Angerer (25). Nonspecific hybridization was controlled by parallel hybridizations on serial sections with a sense strand RNA probe. Autoradiography was carried out at a 7-d interval and the developed slides were counter-stained with methylene green.

CAT plasmid construction, DNA transfection, and CAT assay. The 5′-flanking region (between +1 and −374) of the human TGF-α gene was amplified by PCR with two restrictions attached to the two PCR primers (Sph1 to −374 and XbaI to +1). The chimeric construct, namely 374-TGF-α/CAT3, was prepared by inserting the amplified TGF-α promoter region to the promoterless plasmid pBLCAT3 upstream of the CAT gene between SphI and XbaI sites. The expression plasmids encoding various retinoic acid receptors (RAR-α, -β, -γ) were generously provided by Dr. Pierre Chambon (IGBMC, Illkirch, France). The pCH110 plasmid, encoding β-galactosidase cDNA which is under the control of SV40 promoter, was used in co-transfection as a control for normalizing the transfection efficiency. DNA transfection was carried out in a Lipofectin™-mediated method according to the standard procedure suggested by the manufacture (GIBCO BRL, Gaithersburg, MD). Briefly, each 60-mm culture dish at 70–80% confluence in the absence of vitamin A was washed once with the serum-free F12 medium, then transfected with a 0.5 ml of lipid/DNA mixture that contained 3 μg of TGF-α/CAT3, 1 μg of pCH110 plasmid DNA, and 1 μg of RAR expression plasmid DNA or the control pUC18 plasmid DNA. Six to eight hours later, an additional 0.5 ml of serum-free, vitamin A–free culture medium was added to each dish, and the medium was changed the next day in the presence or absence of vitamin A (all-trans-retinol, 0.1 μM) (Sigma Chemical Co., St. Louis. MO). Cultures were harvested 2 d later. The cells were scrapped from the plates and cell extracts were prepared by three cycles of freeze-thaw in 0.25 M Tris-Cl (pH 8.0). The CAT assays were performed in 0.25 M Tris-Cl (pH 8.0), 2 μl of ^{14}C-chloramphenicol (0.025 mCi/ml) (ICN, Irvine, CA), and 5 μl of n-butyryl coenzyme A (5 mg/ml) (Promega). The CAT activities were analyzed by liquid scintillation counting (33) and normalized with the β-galactosidase activity for each transfection. The β-galactosidase activity was determined by Promega's β-galactosidase enzyme assay system, using the protocol suggested by the manufacturer.

Results

Quantitative TGF-α message levels in TBE culture. To examine vitamin A–regulated expression of the TGF-α gene in tracheobronchial epithelium, we extracted RNA from human and non-human primate TBE cell cultures maintained with or without retinol, and subjected them to RNA northern blot analysis. By this method, no visible signals were detected on autoradiograms, with up to 50 μg of total RNA from TBE cells (data not shown). Because of this apparent low level of expression of TGF-α, three approaches were undertaken. First, a RT-PCR method was used. As shown in Fig. 1 *A*, RNA obtained from the retinol-treated TBE cultures failed to produce a 350-bp TGF-α cDNA fragment (Fig. 1 *A*, lane *1*), while RNA from the untreated culture did (Fig. 1 *A,* lane *2*). This difference is apparently not due to the quality of RNA since

1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; RARE, retinoic acid responsive element; RPA, RNase protection assay; RT, reverse transcriptase; TBE, tracheobronchial epithelial.

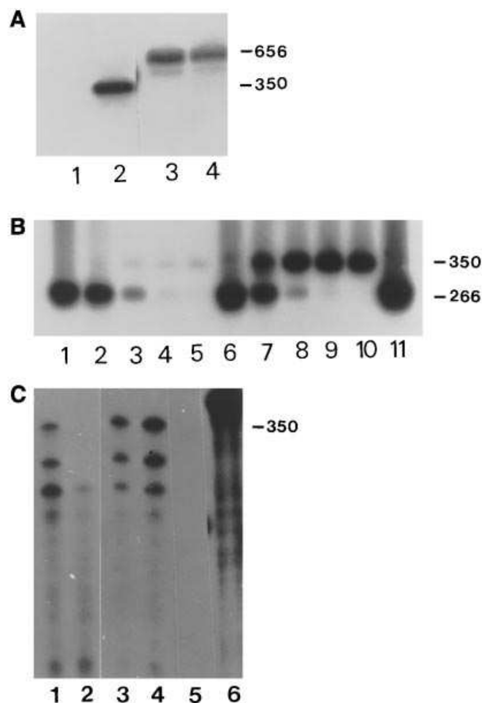

Figure 1. Effect of retinol on TGF-α gene expression in human primary TBE cells and in an immortalized human bronchial epithelial cell line, BEAS-2B cells. (*A*) Characterization of TGF-α and β-actin messages in primary TBE cells in culture. Primary TBE cells were maintained in culture with (lanes *1* and *3*) or without retinol (lanes *2* and *4*) supplement for 7 d as described. RNA were isolated and 0.1 μg of them were used for RT-PCR. Both TA1 and TA2 primers were used for the amplification of TGF-α message, which should generate a 350-bp PCR product, while the amplification of β-actin was based on two commercial primers designated for the generation of 656-bp product. These RT-PCR were carried out in separate tubes. (*B*) Quantitative RT-PCR for TGF-α mRNA in human primary TBE cell cultures. For RT-PCR analysis, 75 ng of total RNA was combined with serial tenfold dilutions of mutant TGF-α cRNA prior to cDNA synthesis and amplification (32). PCR products were separated by electrophoresis in 2% agarose gels. Using both TA1 and TA2 primers, the mutant cRNA template should produce a 266-bp DNA fragment, while the wild-type template is a 350-bp DNA product. DNA was blotted onto Nytran membrane (Schleicher and Schuell) and subsequently hybridized to a ^{32}P-labeled monkey TGF-α probe, pMTA as described in text. Autoradiograms were quantitated by laser densitometry. Lanes *1* and *6*, 100 pg mutant cRNA; lanes *2* and *7*, 10 pg mutant cRNA; lanes *3* and *8*, 1 pg mutant cRNA; lanes *4* and *9*, 0.1 pg mutant cRNA; lanes *5* and *10*, 0.01 pg mutant cRNA were mixed with total RNA for RT-PCR amplification using both TA1 and TA2 primers as described in text. Lane *11* is a control that contained 1-μg mutant cRNA and no experimental total RNA. Lanes *1–5* are cultures treated with retinol, while lanes *6–10*, cultures without retinol treatment. (*C*) Ribonuclease protection assay for TGF-α mRNA in human primary TBE cell cultures and BEAS-2B cells. Human TBE cells (lanes *1* and *2*) and BEAS-2B cells (lanes *3–5*) were cultured with or without the addition of retinol and extracted for total RNA as described above. A ^{32}P-labeled TGF-α cRNA probe, which is slightly greater than 350 bp because of cloning sites, was prepared as described in text. Protection assays were performed on 15 μg of total RNA using a commercially available kit (Ambion). Protected fragments were electrophoretically separated from digested RNA in 6% urea/polyacrylamide gel. Lane *1*, primary human TBE cultures without retinol treatment; lane *2*, primary human TBE cultures treated with retinol; lane *3*, RNA from BEAS-2B cultures treated with retinol; lane *4*, RNA from BEAS-2B cultures without retinol treatment; lane *5*, yeast tRNA control reaction with RNase added; lane *6*, yeast tRNA control reaction with no RNase treatment. The higher molecular weight band in lane *6* represents an undigested probe with additional plasmid sequences because of cloning, which is slightly larger than the actual protected 350-bp fragment. Lower molecular weight bands in lanes *1–4* are partial digests of the 350-bp protected fragments as a result of known nucleotide mismatches between the monkey probe and human TGF-α mRNA.

these same RNAs, in separate tubes, were able to produce 650-bp β-actin cDNA fragments (Fig. 1 *A*, lanes *3* and *4*) when β-actin primers were used. To further evaluate this PCR result, a quantitative RT-PCR method, based on the primer competition, was developed for the quantitation of the TGF-α message level (Fig. 1 *B*). It has been previously demonstrated that the inclusion of a modified template for the gene of interest can successfully control for experimental variation during quantitative RT-PCR (32, 34, 35). Using a similar approach, we constructed a mutant TGF-α template with 266 bp that used the same pair oligonucleotide primers as the wild-type TGF-α which is 350 bp. Quantitation of mRNA was based on competitive, co-amplification of human TBE cell mRNA with cRNA generated from the mutant TGF-α template. This method allows the determination at what amount of the mutant cRNA is needed for each RNA sample when an equal amount of mutant DNA band (266 bp) and wild-type one (350 bp) is generated in this RT-PCR assay. Because of the competitive nature between the mutant and wild type templates on same primers, the amount of mutant cRNA needed for this purpose is proportion to the level of the message of interest in cells (32). By this method, we observed an ∼ 30-fold enhancement of TGF-α message levels in TBE cells cultured under the retinol-deficient condition. To confirm the validity of our competitive RT-PCR results, we also performed ribonuclease protection assays (RPA) to detect TGF-α mRNA in our TBE cell cultures (Fig. 1 *C*). RPA experiments also indicate that the abundance of TGF-α mRNA is lower in TBE cells treated with retinol, compared with cells from retinol-deficient cultures. These analyses at the RNA level are consistent, at least qualitatively, with the protein data presented in our earlier publication (23). Furthermore, a similar RPA result (Fig. 1 *C*) was obtained when a similar experiment was carried out in the immortalized human bronchial epithelial cell line BEAS-2B, clone S.6 (36). Detection of elevated TGF-α gene expression in both primary TBE cell cultures and a bronchial epithelial cell line suggests that this is a general phenomenon of autocrine/paracrine growth in airway epithelial cells associated with a vitamin A deficiency.

In situ hybridization. To further understand this phenomenon, an in situ hybridization technique was used to identify cell types involved in the expression. As shown in Fig. 2 *A*, in the presence of vitamin A, only a few primary cells expressed a significant level of TGF-α message; however, the level of expression is lower than cells maintained in the absence of vitamin A (Fig. 2 *B*). Furthermore, in contrast to the vitamin A-treated culture, > 90% of cultured cells in the absence of vitamin A expressed TGF-α mRNA. It appears that vitamin A has at least two effects on TGF-α gene expression, one is to reduce the abundance of the message, and the other is to reduce the number of cells expressing the message. A similar observation was obtained with the clonal cell line, BEAS-2B, of immortalized human bronchial epithelial cells (data not shown).

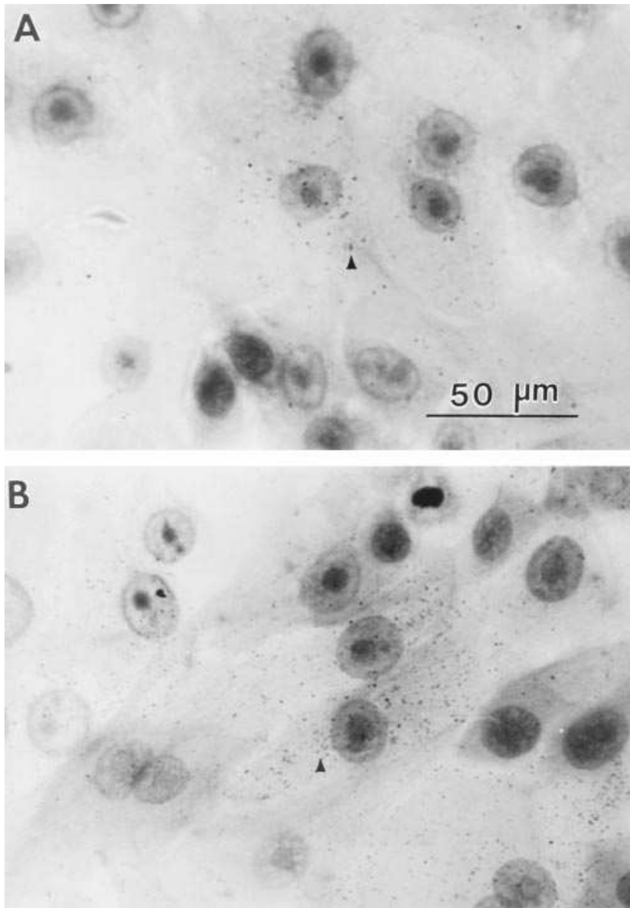

Figure 2. In situ hybridization of TGF-α mRNA in primary human TBE cells grown in culture. Both ^{35}S-labeled sense and antisense TGF-α cRNAs were generated from the monkey TGF-α cDNA clone, pMTA, using the cRNA generating kit from Ambion Inc. Each slide was hybridized with about 1x10^6 counts of probes in 0.2 ml of hybridization solution as described (25). An autoradiogram was developed after seven days and slides were counter-stained with methylene green. (*A*) Primary culture maintained in the vitamin A-supplemented medium; (*B*) Primary culture maintained in the medium without the supplement of vitamin A. Positive hybridization grains are indicated by arrowheads. The control, a sense cRNA probe, yields no positive hybridization (data not shown). Bar, 50 μm.

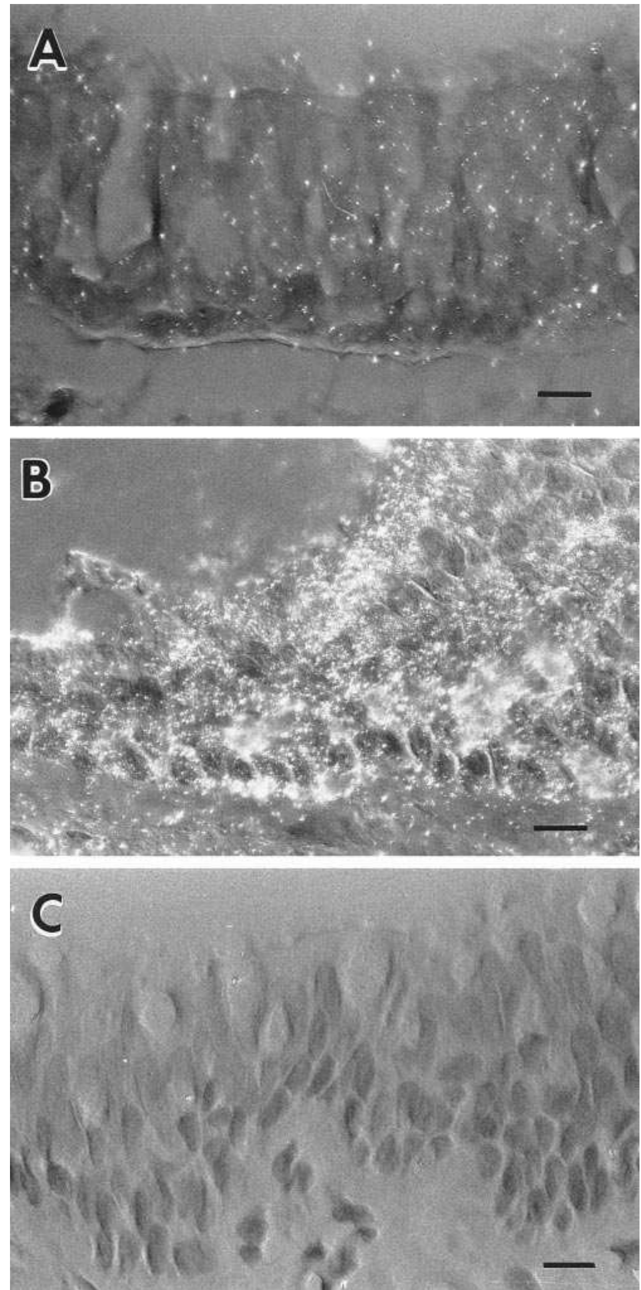

Figure 3. Localization of TGF-α expression in histological sections of monkey tracheobronchial explants by in situ hybridization. Tracheobronchial explants were cultured for two weeks in hormone-supplemented, serum-free medium, with 0.1-μM retinol (*A* and *C*) or without 0.1-μM retinol (*B*) as described in the text. Paraffin sections (*A* and *B*) of tracheal explants were hybridized with an antisense ^{35}S-labeled monkey TGF-α RNA probe, generated from pMTA as described in Fig. 2, overnight at 55°C and processed as described by Angerer and Angerer (25). Nonspecific binding was controlled by parallel hybridizations on serial sections using a sense stranded RNA probe (*C*). Bar, 10 μm.

To address whether such an in vitro cell culture phenomenon also occurs in vivo in airway epithelium, we carried out the in situ hybridization on paraffin sections of monkey tracheobronchial explants that were maintained either in the absence or in the presence of vitamin A (Fig. 3). Organ cultures were used since differentiated features could be maintained in vitro and the vitamin A manipulation was easier than in vivo. Under the serum-free experimental conditions, similar to the primary cultures of TBE cells, tracheobronchial explants cultured with retinol for 14 d maintained an intact pseudostratified epithelium (Fig. 3 *A*). Tracheobronchial explants cultured in a retinol-deficient medium resulted in patchy squamification of the epithelium (Fig. 3 *B*). This sporadic distribution is characteristic of squamous metaplasia of the bronchus in vivo (9). In situ hybridization of paraffin sections from these tracheobronchial explants indicated a very low level of TGF-α specific hybridization within the epithelium of retinol supplemented cultures (Fig. 3 *A*). Positive hybridization was localized in the basal and ciliated cell types. In contrast, higher levels of TGF-α specific hybridization were observed within the epithelium of explants cultured under retinol-deficient conditions (Fig. 3 *C*). The

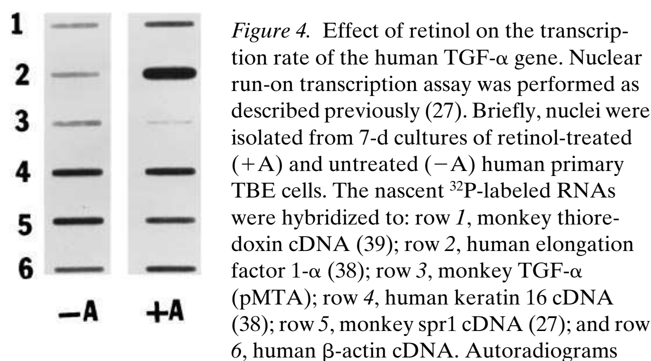

Figure 4. Effect of retinol on the transcription rate of the human TGF-α gene. Nuclear run-on transcription assay was performed as described previously (27). Briefly, nuclei were isolated from 7-d cultures of retinol-treated (+A) and untreated (−A) human primary TBE cells. The nascent ^{32}P-labeled RNAs were hybridized to: row *1*, monkey thioredoxin cDNA (39); row *2*, human elongation factor 1-α (38); row *3*, monkey TGF-α (pMTA); row *4*, human keratin 16 cDNA (38); row *5*, monkey spr1 cDNA (27); and row *6*, human β-actin cDNA. Autoradiograms were quantitated by laser densitometry. A similar result has been repeated twice with primary cultures derived from different human tissues.

specificity of this hybridization was particularly evident when compared with control slides hybridized with a sense probe (Fig. 3 *C*). These results demonstrate that elevated TGF-α expression in airway epithelium under vitamin A-deficient conditions is clearly associated with the squamous cell phenotype.

Vitamin A downregulates TGF-α gene expression at the transcriptional level. To elucidate the nature of this apparent retinol inhibition of gene expression, a nuclear run-on transcriptional assay was performed to determine whether altered TGF-α mRNA levels occur also at the transcriptional level. Nuclei were isolated from cultured TBE cells grown in retinol-supplemented or retinol-deficient medium. Several control hybridizations were carried out with this study. These controls included; (*a*) the hybridization with cDNA slots of monkey small proline-rich protein gene, spr1 (27, 37); (*b*) human keratin No. 16 (38); (*c*) β-actin, known not to be regulated by vitamin A at the transcriptional level; and (*d*) the cDNA slot known to be up-regulated by vitamin A at the transcriptional level, such as the thioredoxin (39) and elongation factor 1-α cDNAs (38). Using these controls, it was observed that the hybridization signal between ^{32}P-labeled run-on transcripts and the TGF-α cDNA insert was lower in retinol-supplemented cultures, compared with retinol-deficient cultures (Fig. 4). Densitometric analysis of autoradiograms indicated a three-fold enhancement of the transcription of the TGF-α gene when compared with the control's actin gene expression in cultures without the supplement of retinol. It is necessary to point out that at the mRNA level, threefold more message was found in cultured cells grown in the absence of vitamin A than in its presence. This apparent difference between the northern blot hybridization and the nuclear run-on assay suggests that vitamin A may have both transcriptional and post-transcriptional effects on TGF-α gene expression. The nature of the post-transcriptional control remains to be elucidated.

To further elucidate the transcriptional mechanism, a promoter-reporter chimeric construct transfection system was carried out. As shown in Fig. 5, the 374-bp DNA fragment of human TGF-α gene 5′-flanking region contained the necessary elements to direct a transient expression of the reporter gene CAT, compared with the promoterless pBL-CAT3 transfected cells. This relative CAT activity is comparable with the published report (40). However, the relative CAT activity in 374-CAT3-transfected cells was reduced more than fourfold by vitamin A treatment. The co-transfection with various nuclear receptors (RARs) did not alter the relative CAT activity, nor the vitamin A-inhibited phenomenon except in the RAR-α co-transfection, which reduced the relative CAT activity by half in the absence of vitamin A. A similar result was obtained if retinoic acid was used instead of retinol (data not shown). However, the cytotoxicity of retinoic acid on airway epithelial cells is higher than retinol (data not shown).

Discussion

Vitamin A and its retinoid derivatives are important regulators in the maintenance of homeostasis of airway epithelium. In this and a previous report (23), we demonstrated that vitamin A is a potent inhibitor for TGF-α gene expression. The previous results suggested that TGF-α may be involved in the autocrine/paracrine regulation of TBE cell growth in the vitamin A deficient condition (23). This study demonstrated further that the inhibition by vitamin A occurs at the mRNA level, or at least partly at the transcriptional level. This conclusion is based on the analyses of TGF-α message level by a competitive RT-PCR and the RNase protection assay. In both approaches, a 30-fold difference in TGF-α message level was observed between vitamin A–treated and –untreated cultures.

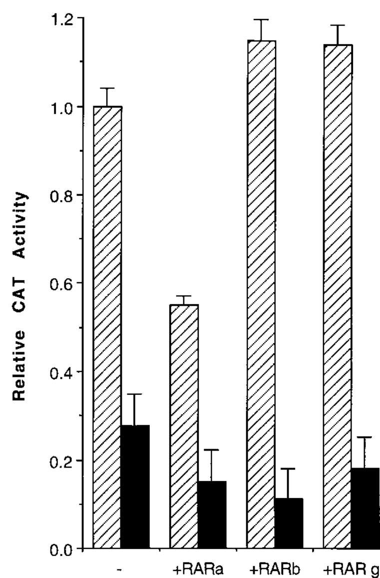

Figure 5. Effect of retinol on TGF-α promoter activity in primary human TBE cells. The chimeric construct 374-CAT3, containing 374 bp of TGF-α 5′-flanking region (40) and the reporter gene CAT, was prepared in a promoterless pBL-CAT3 (27). Primary human TBE cells were maintained in the serum-free hormone-supplemented medium in the absence of retinol. DNA transfection was performed in 35-mm tissue culture dishes, using Lipofectin™ (GIBCO BRL) according to the protocols suggested by the manufacturer. After transfection, half of transfected culture dishes were treated with retinol (*solid bars*) and half were not treated (*hatched bars*). For co-transfection experiments, expression vectors containing RAR α, β, and γ cDNAs were combined with 374-CAT3 at a 1:1 concentration ratio (a total of 2 μg DNA/dish). As a control, 374-CAT3 transfected cells without RAR were co-transfected with equivalent concentrations of PGEM4Z (a total of 2 μg DNA/dish). For normalizing the transfection efficiency, cells were also transfected with 1-μg pSV-β-gal plasmid DNA. At 72 h after transfection, cell extracts were prepared and assayed for CAT and β-galactosidase activities as previously described (27). To account for differences in cell numbers, CAT activity levels were measured relative to β-galactosidase activity of transfected cells per dish, and then normalized with the control cultures treated with retinol alone. Each assay was carried out in triplicate dishes and these data were averaged. Variations among the triplicate were shown to be within 20%. A similar result has been repeated twice with primary cultures derived from different human tissues.

We further extended this result from a primary cell culture system to an organ culture system that continuously maintains the architecture of airway epithelium in vitro. Under this organ culture system, a deprivation of vitamin A causes not only the "squamous cell metaplasia"-like phenomenon but also an enhancement of the expression of TGF-α message in airway epithelium. These results strongly suggest the notion that vitamin A is a negative regulator for TGF-α gene expression.

At present, the precise mechanism of vitamin A-down regulation of TGF-α gene expression has not been completely worked out; however, data present in this communication suggests that at least two mechanisms are possibly involved. We observed that the difference in the mRNA abundance between the vitamin A–treated and –untreated cultures is close to 30-fold, based on the competitive RT-PCR and RNase protection assays. However, the difference in the transcriptional analyses, based on the nuclear run-on and the promoter-reporter transient transfection studies, is three- to fourfold. The difference between the assays suggests that vitamin A may affect both the transcriptional and the post-transcriptional mechanisms involved in the regulation of TGF-α gene expression. The post-transcriptional mechanism is complex. At present, we are unable to carry out the analysis of the TGF-α mRNA stability in culture because of a low abundance of TGF-α message in cultured TBE cells. Therefore, the post-transcriptional regulation mechanism remains to be elucidated.

Based on the results obtained from the nuclear run-on assay and the reporter gene expression, we demonstrated partly the involvement of the transcriptional mechanism. Transcriptional activation normally requires the interaction of an active transcriptional factor(s) with specific *cis*-elements located in the 5′ flanking region of a gene. Numerous retinoic acid response elements (RARE) have been identifed as well as retinoid-binding nuclear receptors (RARs and RXRs) that function as transcriptional factors (41–46). However, nucleotide analysis of a 1.8-kb 5′-flanking region of the human TGF-α gene did not indicate the presence of any reported RARE (40). In an attempt to characterize the mechanisms of retinol regulation of TGF-α mRNA transcription, a chimeric construct was prepared in which a 374-bp domain of the human TGF-α 5′-flanking region was introduced upstream of the reporter gene chloramphenicol acetyltransferase (CAT). This region was initially chosen for study because it has previously been reported to produce the strongest basal promoter activity in Chinese hamster ovary cells (40). When this chimeric construct, 374-CAT3, was transfected into the primary human TBE cells, transient expression of CAT activity was observed (Fig. 5) at levels consistent with previously published reports (40). However, when transfected cells were treated with retinol, CAT activity was reduced by more than fourfold. To enhance this apparent vitamin A response and to characterize the nuclear receptors mediating this event, expression vectors containing coding sequences for RAR α, β, and γ were co-transfected with the TGF-α promoter chimeric construct 374-CAT3 (Fig. 5). In the absence of retinol, the co-transfection with RARs, except RAR-α, had no effect on the relative CAT activity when compared with the control co-transfected with the control pUC18 plasmid DNA. The co-transfection with RAR-α decreased the relative CAT activity by half. However, in every transfection, the presence of retinol further inhibited the CAT activity. These results suggest that the interactions between the retinol metabolites and RARs are either directly or indirectly responsible for the downregulation of TGF-α gene transcription. We have no explanation why only RAR-α co-transfection would decrease the CAT activity. One possibility is that RAR-α is different from other RARs in the binding of retinoid metabolites (47–49) or interactions with other transcriptional factor(s) (50). At least two vitamin A-inhibiting transcriptional mechanisms have been demonstrated. One involves a possible direct interaction between the RARE sites and retinoic acid receptor (RAR/RXRs) (51). The other involves an indirect mechanism through the decrease of the AP-1 activity by vitamin A treatment (52). Further studies at the DNA-protein interaction level will help to resolve the nature of this downregulation mechanism.

High level expression of TGF-α has been previously associated with the malignant cell phenotype, yet there is growing evidence to suggest it is more strongly correlated with benign hyperproliferative events (53, 54). The data presented here indicates a putative role for TGF-α in squamous cell differentiation; a recent report demonstrating immunohistochemical detection of TGF-α in squamous metaplasia of normal human airways lends support to this idea (55). Consistent with this notion, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a potent chemical for the induction of squamous cell differentiation in airway epithelium (12), was shown to enhance TGF-α mRNA accumulation and TGF-α protein secretion in cultured human keratinocytes (56). Taking this evidence together, we conclude that the expression of TGF-α is closely associated with the development of squamous epithelium and that vitamin A can effectively reverse this phenotype by downregulating the expression of this gene at the mRNA level.

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