Inhibition of Epidermal Growth Factor-like Growth Factor Secretion in Tracheobronchial Epithelial Cells by Vitamin A

Lisa A. Miller, Ling Zhong Cheng, and Reen Wu

California Regional Primate Research Center and Department of Internal Medicine, University of California at Davis, Davis, California 95616

ABSTRACT

Vitamin A deficiency of respiratory tract epithelium results in the phenomenon of squamous cell metaplasia. The mechanisms by which vitamin A regulates airway epithelial cell growth and differentiation are not completely understood. In this study, we focused on the effects of vitamin A (retinol) on growth of human and non-human primate tracheobronchial epithelial (TBE) cells in culture. Retinol and its derivatives have little growth-stimulatory effect on TBE cells that are maintained in primary culture in a serum-free medium supplemented with 6 hormonal supplements: insulin, transferrin, epidermal growth factor (EGF), hydrocortisone, cholera toxin, and bovine hypothalamus extract. However, it was observed that retinol exhibited dose-dependent inhibition of TBE cell growth when EGF was removed from this serum-free culture condition. This inhibition can be reversed if I I.I or the conditioned medium of primary TBE cells that are maintained in vitamin A-deficient condition is added. This type of EGF-retinol interacting phenomenon was not observed with the 5 remaining hormonal supplements. Analysis of 125I-labeled EGF binding shows a down-regulation of the high affinity binding sites (Kd = 0.09 nm) on TBE cells grown in the absence of vitamin A. These results suggest that TBE cells are capable of secreting an EGF-like growth factor in the absence of vitamin A. The possibility that transforming growth factor-α (TGF-α) is involved in this phenomenon is further examined by antibodies specific to TGF-α and its binding to an EGF-receptor. Using the TGF-α antibody, the presence of a TGF-α-specific antigen was found to be 3-fold higher in the conditioned medium obtained from the vitamin A-deficient cultures than that derived from retinol-treated cultures. Furthermore, the antibody neutralizing the TGF-α binding to an EGF receptor was able to reduce the DNA synthesis associated with the vitamin A deficiency. These results suggest that vitamin A plays an important regulatory role in the paracrine/autocrine secretion of EGF/TGF-α-like mitogen in TBE cell cultures.

INTRODUCTION

In conducting airway epithelium, the importance of vitamin A in the regulation of the normal mucociliary functions is well recognized (1). Both in vitro and in vivo studies demonstrated a reversible squamous cell metaplasia of airway epithelium as a result of vitamin A deficiency (1-3). The nature of this change is currently unclear. A similar lesion of squamous cell metaplasia also occurs in the airways after carcinogen exposure (4). It has been suggested that squamous cell metaplasia may represent an early event in the development of bronchogenic cancer (5). Although a direct, causal relationship between vitamin A deficiency and neoplasia has not been demonstrated, indirect evidence supports such a proposal. For example, certain vitamin A derivatives (retinoids) can inhibit the development of chemically induced bronchogenic cancers in experimental animals (6-8). Also, epidemiological studies have suggested a correlation between vitamin A deficiency and increased risk of lung cancer (9, 10).

The mechanism by which vitamin A regulates the life cycle of the airway epithelium is currently unclear. Vitamin A may serve as a direct growth-stimulating or -inhibiting factor on certain airway cell types. McDowell et al. (11-13) have demonstrated that vitamin A is important for maintaining normal growth, shape, and differentiation of hamster tracheal secretory cells. We recently found that the maintenance and proliferation of mucin-secreting cells in human and monkey TBE cultures is vitamin A dependent. Evidence to support a negative role for vitamin A on airway cell growth is still incomplete. However, it has been demonstrated that vitamin A deficiency initially results in a basal cell hyperplasia within airway epithelium, followed by squamation by the process of (2, 3). In the serum-free medium developed in this laboratory, the growth of protease-dissociated human TBE cells is not significantly altered by vitamin A, despite a significant increase in mucus cell population (14).

Alternatively, instead of serving as a growth-stimulating or -inhibiting factor, vitamin A may function in a regulatory manner by maintaining a balance between both positive and negative growth factors in airway epithelium. A deficiency of vitamin A may alter the equilibrium within this microenvironment such that hyperplasia of basal cells is favored. In contrast, the presence of vitamin A may inhibit this hyperplasia and enhance mucous cell differentiation. This notion is supported by the recent study that demonstrates that an exogenous treatment of fetal lamb tracheobronchial epithelium with retinol and EGF in vivo induces premature mucous cell differentiation (15). In this communication, we focus on the potential regulatory role of vitamin A in the alteration of growth factor responsiveness by airway epithelial cells. This study was carried out in a serum-free, hormone-supplemented, defined medium developed for culturing airway epithelial cells dissociated from various animals and human airway tissues (16). We observed that the growth response of human and non-human primate TBE cells to EGF is altered by the presence or absence of vitamin A. In conjunction with these experiments, we demonstrated that the secretion of an EGF-like growth factor is apparently inhibited by vitamin A. In addition, we present evidence that suggests that TGF-α is a good candidate for this putative growth factor.

MATERIALS AND METHODS

Materials. F12 and minimal essential medium were purchased from GibCO-BRL (Grand Island, NY). Ins, Tt, HF, dexamethasone, and all-trans-retinol were products of Sigma Chemical Co. (St. Louis, MO). Both culture and receptor grades of EGF were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). CT was obtained from List Biological, Inc. (Campbell, CA). Bovine hypothalami were obtained from PelFreeze (Pine Bluff, AR), and the extract was prepared as described previously (17). Purified human recom-

Received 12/23/92; accepted 3/23/93.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by grants from the NIH (HL35635, ES00628), the Council for Tobacco Research, Inc. (Grant 2611), and the California Tobacco-Related Disease Research Program (RT-0409).

2 Present address: Department of Histology & Embryology, Shanghai Medical School, Shanghai, 200032, People's Republic of China.

3 To whom requests for reprints should be addressed, at California Regional Primate Research Center, University of California at Davis, Davis, CA 95616.

4 The abbreviations used are: TBE, tracheobronchial epithelial (cells); EGF, epidermal growth factor; TGF-α, transforming growth factor-α; Ins, insulin; Tt, transferrin; HF, hydrocortisone; CT, cholera toxin; BHE, bovine hypothalamus extract; PBS, phosphate buffered saline; BSA, bovine serum albumin; CM, conditioned medium; RIA, radioimmunoassay.

5 Y. H. Zhao and R. Wu, manuscript in preparation.
binant TGF-α, mouse anti-human TGF-α monoclonal antibody, and the neutralizing antibody of TGF-α binding were obtained from Oncogene Science (Manhasset, NY). 32P-labeled goat anti-mouse IgG, [125I]KI, and [3H]thymidine (60 Ci/mmol) were obtained from ICN (Irvine, CA). Falcon dishes were obtained from Fisher Scientific (Santa Clara, CA). Centrifuge-3 microconcentrators were obtained from Amicon (Danver, MA). The other chemicals used in this study are the highest quality products commercially obtainable.

Sources of Tissues and Cell Culture. Human tracheobronchial tissues were obtained from organ donor patients or autopsies through the University of California, Davis, Medical Center (Sacramento, CA) or from International Institute for Advance Medicine, Inc. (Exton, PA). Rhesus monkey tracheobronchial tissues were obtained from the California Regional Primate Research Center of University of California at Davis. Within 24 h after excision, tissues were immersed in minimal essential medium, kept at 4°C, and immediately sent to our laboratory. TBE cells were isolated by a protease method described previously (14, 16).

Protease-dissociated TBE cells were plated on Falcon plastic culture dishes at a cell density of 1 x 10³ cells/cm² in serum-free F12 medium supplemented with Ins (5 µg/ml), Tf (5 µg/ml), EGF (20 ng/ml), CT (10 ng/ml), HC (1 µm), or dexamethasone (0.1 µm), and BHE (30 µg/ml), as described previously (15, 17). This complete medium is referred to as 6F medium. When one of these 6 hormonal supplements, such as Ins or EGF, was omitted from this medium, the medium is referred to as 5F (−Ins) or 5F (−EGF), respectively. The parentheses indicate the individual hormonal supplement omitted. To investigate the effect of vitamin A on cell growth, all-trans-retinol was added to the medium at 0.1 µm or as indicated in each experiment. Since we previously observed that all-trans retinoic acid (Sigma) is potentially more toxic to cells than retinol (14), we used all-trans-retinol for the vitamin A treatment experiments. The retinol-treated cultures are thereby referred to as 6F+A, 5F (−Ins)+A, or 5F (−EGF)+A, etc. Unless it is specified, the medium change was carried out every other day. Cell number in each dish was determined electronically by a Coulter cell counter (Coulter Electronics, Inc., Hialeah, FL) after dissociating cells from culture dishes with trypsin-EDTA.

125I-Labeled EGF Binding Assay. To characterize EGF receptors, receptor grade EGF was labeled with 125I by the chromium-T method (18) and purified as described previously (19). Specificity was at 1–5 x 10⁴ cpm/ng protein. Binding was carried out at 4°C for 3 h on 35-mm tissue culture dishes containing approximately 5 x 10⁴ cells/dish. After binding, dishes were washed 6 times (1 ml each) with ice-cold PBS, lysed in 0.1 M NaOH, and counted in a Micromedic Systems rack gamma counter (ICN, Irvine, CA). Nonspecific binding was determined by treating some dishes with 1000-fold excess unlabeled EGF prior to the binding assay, and the radioactivity of nonspecific binding was subtracted from each binding assay. Calculations of EGF-binding sites and kinetics of binding were based on the Scatchard plot analysis (20).

Quantification of TGF-α-like Antigen in Culture Medium. A RIA was developed to quantit TGF-α-like antigen in the CM of primary TBE cell cultures. For the preparation of CM, the 7-day-old TBE cultures maintained with or without retinol were washed twice with F12, then changed to 5F (−BHE) or 5F (−BHE)+A medium, respectively. BHE was removed from the incubating medium because of a low level of TGF-α-like antigen contamination. After a 24-h incubation, CM were collected and concentrated 20-fold by a Centrifuge-3 microconcentrator (Amicon, Danver, MA). Concentrated CM were then dried onto microtiter wells, and the wells were further coated with a bicarbonate-based buffer (pH 9.2). Wells coated with standard TGF-α at different amounts (2.5–75 ng/well) were prepared in a similar way. These wells were pretreated with 5% BSA in PBS, and TGF-α antigen was detected by a mouse anti-human TGF-α monoclonal antibody (0.1 µg/ml), purified by 125I-labeled goat anti-mouse IgG as a secondary antibody. After an extensive wash with BSA-PBS, the radioactivity in each microtiter well was determined in a Micromedic System rack gamma counter. The concentrations of TGF-α in samples were determined by comparison with a standard curve generated by microtiter wells coated with various amounts of reference TGF-α.

Neutralization of TGF-α Activity. Primary TBE cells were plated on 24-well plates in 6F and 6F+A medium. After 24 h, these cultures were changed to 4F (−EGF, −BHE) and 4F (−EGF, −BHE)+A media, respectively, but supplemented with 0.15% BSA. After 3 more days of incubation, an anti-serum specific for preventing the TGF-α binding to EGF receptor (21) was added to these cultures at 0.1 µg IgG/ml. Forty-eight h later, DNA synthesis was measured by a 2-h incorporation of [3H]thymidine (5 µCi/well). Radioactivity of the acid-insoluble fraction for each well was measured in a Beckman LS3801 liquid scintillation counter (Fullerton, CA).

RESULTS

Effects of Retinol on the Growth Response of TBE Cells to Individual Hormonal Supplements. On plastic tissue culture surface, protease-dissociated human TBE cells required the aforementioned 6 hormonal supplements (6F) for proliferation in culture. This requirement was demonstrated by a decrease in the cell number of 7-day-old primary TBE cultures that were maintained in medium deficient in 1 of the 6 hormone supplements (Fig. 1). Among these 6 hormonal supplements, Ins, Tf, HC, and BHE were found to be important for cell growth, since their respective omissions resulted in a more than 50% reduction in cell number. The omission of CT slightly decreased cell number (30%). These reductions were consistently found in primary cultures derived from different human donors, including patients of cystic fibrosis (Fig. 1, a and b). The addition of retinol to these cultures had little effect on the growth response of TBE cells to these 5F (minus one hormonal supplement) media. In contrast, retinol treatment had a dramatic effect on growth of TBE cells in

![Fig. 1. Effects of retinol and various growth factors/hormones on growth of human TBE cells in primary cultures. Protease-dissociated TBE cells were prepared as described in "Materials and Methods." Cells were plated on tissue culture dish at a density of 5 x 10⁴ cells/cm² in complete 6F medium or in 5F medium deficient in one of the growth factors/hormones as indicated. At day 2 after plating, media were changed and retinol (0.1 µM) was added to half of culture dishes (U), and the other half was maintained in the vitamin A-free conditions (D) in the same hormonal supplemented media. At day 7 after plating, cell number in each dish was counted as described in text. Average data were obtained from triplicate dishes for each condition, and the variations (data not shown) between the triplicate dishes were within 15% of the average number. A, primary TBE cells obtained from female non-CF donor at age 65; B, primary TBE cells obtained from male CF donor at age 14.](http://cancerres.aacrjournals.org)
EGF-deficient medium. In the absence of retinol, the omission of EGF did not result in a large decrease in cell number. In primary cultures derived from 2 different donors, one had a 15% reduction in cell number (Fig. 1a), and the other had no reduction (Fig. 1b) in 5F-(-EGF) medium. However, in the presence of retinol, the omission of EGF from the culture medium consistently resulted in a greater than 75% reduction of cell numbers. This retinol-dependent paradoxical phenomenon was not observed in the 5 remaining hormonal supplements. These results have been repeated in more than 10 primary cultures of human TBE cells as well as in more than 3 primary TBE cultures of nonhuman primates (data not shown).

Significant morphological differences within cell cultures could also be observed, depending on the presence or absence of retinol and/or EGF. As shown in Fig. 2, a and b, when primary TBE cells are cultured in media without retinol supplementation, the presence (Fig. 2a) or absence (Fig. 2b) of EGF results in little change in cell density and cell morphology. When primary TBE cells are cultured in media with retinol supplementation, the presence (Fig. 2c) or absence (Fig. 2d) of EGF produces a significant reduction in cell density and morphology such as reduced cell contact and plasma membrane blebbing.

The effect of retinol on the EGF-dependent growth stimulation was further examined in a dose-response study. As shown in Fig. 3, a dose-dependent growth inhibition by retinol could be observed in EGF-deficient cultures. The inhibition was 50% at 10 nM and maximal at 100 nM retinol supplementation. Concentration-dependent inhibition of growth could also be observed when other derivatives of retinol such as retinal and retinoic acid were used (data not shown). These experiments have been repeated in 2 other primary cultures of different human donors.

The absolute growth requirement for EGF in the retinol-treated cultures was further established in a dose response study. Following a similar experimental protocol as described in Fig. 1, cell numbers of 7-day-old primary cultures maintained in retinol-supplemented medium were increased by EGF in a concentration-dependent manner (Fig. 4). The addition of less than 2 ng/ml of EGF to retinol-treated

Fig. 3. Dose-dependent effects of retinol on growth of human TBE cells in 5F(-EGF) medium. TBE cells were plated on plastic culture dishes at a density of 2 x 10^4 cells/cm^2 in 5F(-EGF) medium as described in text. Retinol at the indicated concentrations was added at day 1 after plating. As a control, dishes without retinol were treated with 0.01% dimethylsulfoxide (the solvent for dissolving retinol). Cell numbers were determined at day 7 after plating and averaged from triplicate dishes for each condition. Variations (data not shown) between dishes were within a 15% range. Similar inhibitory results were obtained with retinal and retinoic acid, derivatives of retinol (data not shown).

Fig. 2. Phase contrast micrographs of human TBE cells grown in various culture media. Human TBE cells were isolated and plated as described in Fig. 1. At day 7 after plating, these micrographs were taken with a Nikon inverted phase-contrast microscope. Bar, 20 µm. The culture media used in this study were: a, 6F; b, 5F(-EGF); c, 6F+A; d, 5F(-EGF)+A, as described in text.
INHIBITION OF EGF-LIKE SECRETION BY VITAMIN A

47,000 high affinity binding sites/cell were seen in TBE cells cultured in retinol-supplemented medium. In contrast, very few high affinity binding sites were observed in TBE cells in retinol-deficient medium. The low affinity binding sites were apparently not affected by the culture conditions, with or without retinol treatment. Similar experiments have been carried out in 2 other human primary TBE cultures. These experiments demonstrated a similar trend in decreasing the high affinity binding sites for EGF in TBE cells cultured without retinol supplementation.

**Secretion of EGF-like Growth-promoting Activity in Cultures without Retinol Supplementation.** A simple explanation for the down-regulation of EGF receptors is that TBE cells, cultured under a vitamin A-free condition, produce EGF-like growth factor. Secretion of an EGF-like mitogen could explain why TBE cells, cultured under a vitamin A-free condition, do not respond to EGF and have a down-regulation phenomenon on the high affinity EGF-binding sites. To investigate this possibility, we analyzed the putative growth-stimulating activity in CM of TBE cultures. CM from both 5F(−EGF) and 5F(−EGF)+A cultures were prepared and their growth-promoting activities on TBE cells were determined in a primary culture maintained in the 5F(−EGF)+A medium. As shown in Fig. 6, a significant growth-stimulating activity was observed in the CM of the 5F(−EGF) culture but not from the CM of the 5F(−EGF)+A culture (Fig. 6). At the 30% (v/v) levels, the CM of the 5F(−EGF) culture produced an approximately 250% increase in cell number, whereas the same level CM of the 5F(−EGF)+A cultures produced only a 10% increase.

---

**Down-Regulation of EGF Receptor in Cultures without Retinol Supplementation.** The inability of TBE cells in the absence of retinol to respond to EGF growth stimulation was further examined at the receptor level. Using 125I-labeled EGF, the kinetics of EGF binding were studied in cultures treated with or without retinol. As shown in Fig. 5, Scatchard analysis indicated that TBE cells cultured under retinol supplementation exhibited 2 types of binding sites: a high affinity binding site ($K_{d} = 0.09$ nm) and a low affinity binding site ($K_{d} = 1.0$ nm). Based on Scatchard plot, it was estimated that nearly
In a similar manner, the kinetics of growth-stimulating activity of the CM of the 5F(-EGF) culture were studied. Primary human TBE cells were plated at low cell density (1 × 10^3 cells/cm²) in 5F(-EGF) medium. At day 8, these cultures were treated with retinol, EGF, or the CM of the 5F(-EGF) cultures (30%, v/v). The addition of EGF to cultures did not produce significant growth stimulation or inhibition (Fig. 7). In contrast, the addition of retinol to cultures resulted in a reduced cell-proliferation rate. This decrease was observed after only 2 days of incubation, suggesting a deprivation of growth-stimulating activity by retinol treatment. However, this retinol-dependent inhibition could be prevented if EGF, or to a lesser extent the CM of the 5F(-EGF) cultures, was provided. These results suggest that the CM of the 5F(-EGF) cultures can partially substitute for EGF in TBE cultures treated with retinol.

**Secretion of TGF-α Specific Antigen in TBE Cultures.** These preliminary characterizations suggested that the putative growth-stimulating activity in the CM of the 5F(-EGF) cultures has the characteristics of an acidic protein(s). The possibility that growth-stimulating activity in the CM of the 5F(-EGF) cultures may be attributed to TGF-α, an EGF-like protein, was evaluated. A RIA was developed to quantify the amount of TGF-α antigen in CM preparations. Using purified human recombinant TGF-α, a linear standard curve was produced (data not shown). Based on this standard curve, the amount of TGF-α antigen produced by TBE cells cultured in 5F(-EGF) medium was 6.4 ng/day/million cells, while the production was 3-fold less by cells treated with retinol (Table 1). These experiments have been repeated in 2 other primary cultures of different tissue donors.

To further evaluate the functional role of the TGF-α antigen produced in TBE cultures, the neutralizing antibody that prevents TGF-α binding to the EGF receptor was used. As measured by incorporation of [3H]thymidine to the acid-insoluble fraction, treatment with 0.1 μg IgG/ml neutralizing antibody reduced the DNA synthesis activity by 50% in the retinol-free cultures, whereas the same amount of neutralizing antibody had no effect in retinol-treated cultures (Fig. 8). Increasing the concentration of neutralizing antisera in cultures did not result in further inhibition of [3H]thymidine incorporation (data not shown). This result supports the notion that TGF-α antigen secreted by TBE cells cultured in 5F(-EGF) medium may participate in the growth-stimulating activity.

### Table 1 Quantitation of TGF-α Antigen in CM of Monkey TBE Cell Cultures

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>125I-bound (cpm/million cells)</th>
<th>TGF-α produced (ng/million cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With retinol</td>
<td>682 (278)</td>
<td>2.1 (0.85)</td>
</tr>
<tr>
<td>Without retinol</td>
<td>1720 (199)</td>
<td>6.4 (0.74)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses, statistically significant SD, p < 0.05.*

---

![Fig. 7. Effects of CM and EGF on the growth of human TBE cells that have been maintained in 5F(-EGF)+A medium for 8 days. TBE cells were plated at 1 × 10^3 cells/cm². At day 8 after plating, dishes were washed and replaced with the following culture media: 5F(-EGF) ( ), 4F (with EGF) ( ); 4F (with EGF)+A ( ); 5F(-EGF)+A ( ); and 5F(-EGF)+A supplemented with CM (30%, v/v) derived from TBE cell cultures grown in 5F(-EGF) medium as described in Fig. 6 ( ). Cell numbers were determined thereafter at 2-day intervals, and each determination was averaged from duplicate dishes. Bars, variations in duplicate dishes.

![Fig. 8. Effects of TGF-α neutralizing antibody on DNA synthesis of monkey TBE cells cultures. Monkey TBE cells were plated in 6F medium. At day 1 after plating, medium was changed to 4F(-EGF), -BHE) with or without retinol as indicated (+ or + ). At day 3 after plating, 0.1 μg/ml of TGF-α neutralizing antibody (AB) was added to each dish. At 48 h after the addition of antibody, DNA synthesis was measured by the incorporation of [3H]thymidine (5 μCi/ml) into the acid-insoluble fraction. The incorporation was carried for 8 h. Points, mean of quadruplicate dishes.](chart)
**DISCUSSION**

Vitamin A and its retinoid derivatives are important regulators in the maintenance of the homeostasis of airway epithelium. Previously, a majority of the studies have emphasized the role of vitamin A in maintaining mucociliary differentiation (22). Very little attention has been paid to potential mechanisms for regulating cell proliferation by vitamin A. In this communication, the effects of retinol on human and nonhuman primate TBE cell proliferation were examined. It was demonstrated that retinol, as well as its metabolites, retinal and retinoic acid, had little growth-stimulating or -inhibiting activity on TBE cells cultured in serum-free medium supplemented with 6 growth factors/hormones (14). However, when EGF, one of these 6 hormonal supplements, was omitted from this serum-free medium, the addition of retinol to TBE cultures dramatically reduced cell proliferation. This inhibition occurred not only in a dose-dependent manner but also at a level (10–100 nM) close to the physiological level of retinol. This phenomenon was not observed with the 5 remaining hormonal supplements, which include Ins, Tf, HC, CT, and BHE.

One explanation for this phenomenon is that the EGF receptor in TBE cells changes after the retinol treatment. Changes in EGF binding by vitamin A have been demonstrated in several established cell lines (23–26). Most of these changes involve an increase in the EGF receptor numbers but not the affinity. However, the biological significance of this alteration has not been completely resolved. EGF receptors, especially the high affinity binding sites, in several cell cultures tend to decrease when cultures reach their confluency (27). We are able to rule out this possibility by carrying out the binding assay at the same cell density (5 × 10^5 cells/dish). We observed that primary human TBE cells in the presence of retinol exhibited 2 types of EGF binding, high affinity for 125I-labeled EGF (Kd = 0.09 nm) and low affinity (Kd = 1 nm). In contrast, the high affinity binding sites were either reduced or absent in TBE cultures treated with retinol. The presence of the high affinity EGF binding sites in TBE cells supports a greater role of interactions between EGF and its receptor in promoting cell proliferation (28). However, such a notion is not completely supported by data in previous publications. For instance, our laboratory has demonstrated in HeLa cells that the EGF receptor number increased by HC treatment (19). However, this increase had no effect on the manner of EGF dose-dependent growth stimulation in this cell line. A similar phenomenon was also observed in the human epidermal cell line A431. This cell line has a 4-fold higher number of EGF receptors (28) as compared with various other cell lines, yet the growth of A431 cells was inhibited by EGF at a nanogram level (29, 30).

Since changes in EGF binding cannot completely explain why exogenous EGF has no growth-stimulating activity on TBE cells cultured in a vitamin A-free medium, this leads to the current proposal that perhaps retinol treatment can alter the autocrine/paracrine regulatory mechanism of TBE cells (31). This proposal is supported by data presented in Figs. 6 and 7, which show that the CM of retinol-deficient TBE cultures contains growth-promoting activity that can be used interchangeably with EGF to stimulate growth of TBE cells maintained in retinol-supplemented medium. The autocrine/paracrine regulatory mechanism is also consistent with the data of Figs. 4 and 5, which show EGF dose-dependent growth stimulation and high affinity binding sites for 125I-labeled EGF in TBE cultures treated with retinol, but not with cultures without the treatment.

We have, without success, attempted to purify this growth-promoting factor or factors by standard protein purification methods. Because the CM derived from retinol-deficient cultures produced mitogenic activity similar to that of EGF, we chose to study EGF homologues as candidate growth factors. A number of related proteins have been categorized as “EGF-like,” including TGF-α, amphiregulin, and the vaccinia virus growth factor precursor (32). All members of this group exhibit the ability to bind to the EGF receptor, albeit at varying affinities. TGF-α was initially characterized as a growth factor expressed only in transformed and tumorigenic cells (33), but it has now been shown to be expressed at very low levels in a number of different normal adult tissues, including certain regions of the brain, macrophages, and keratinocytes (34–36). Unlike amphiregulin, TGF-α appears to use only the same receptor as EGF and is almost interchangeable in vitro with EGF in normal cellular responses. A distinct exception to this rule exists in primary lung carcinoma cells in which TGF-α enhances, but EGF inhibits, cell proliferation (37). Furthermore, the overexpression of TGF-α has been correlated with a number of squamous cell carcinomas and adenocarcinomas of the lung (38, 39). For these reasons, we chose to pursue TGF-α as the candidate growth factor in our system.

The mitogenic properties of TGF-α are generally attributed to a secreted, 50-amino acid peptide derived from a 160-amino acid transmembrane precursor. Our CM experiments suggest that the enhanced TBE cell proliferation observed under 5F(-EGF) culture conditions [as compared with 5F(-EGF)+A cultures] were the result of an endogenously produced and secreted “factor.” We have shown by RIA that cultures that are deficient in retinol secrete elevated levels of the TGF-α antigen. Because a serum-free medium that was deficient in EGF and bovine hypothalamus extract was used, we are confident of the specificity of the immunoreactivity. Furthermore, a neutralizing antibody to TGF-α was capable of inhibiting growth-stimulating activity in retinol-deficient cultures (Fig. 9). These results suggest that TGF-α or a TGF-α-like peptide is at least partially responsible for the growth-promoting activity present in retinol-deficient TBE cultures. When we initiated the cell culture studies, purified TGF-α protein was not generally available; therefore, we were unable to study the effect of adding TGF-α directly to retinol-supplemented TBE cell cultures. Regardless, we would not be able to unequivocally confirm that the secreted, growth-promoting factor is TGF-α alone, because vitamin A deficiency can induce or inhibit the expression of other genes that may work in conjunction with this mitogen. Interestingly, studies of EGF receptor binding capacity in other mammalian cell systems similarly indicate a concomitant loss of high affinity EGF binding sites as levels of cellular TGF-α secretion increase (40–42).

In conclusion, we demonstrated a vitamin A-regulated autocrine/paracrine phenomenon in primary TBE cell cultures. This phenomenon was observed in both human TBE cultures and monkey TBE cells (data not shown), indicating that the growth-promoting response to vitamin A deficiency is an intrinsic property of airway epithelium. The data presented in this communication suggest that either TGF-α or an analogue may be responsible for the growth-promoting activity in vitamin A-deficient, EGF-deficient TBE cell cultures. As previously described, there is a stage of epithelial cell hyperplasia that precedes the morphological changes that are normally associated with squamous cell differentiation in airway epithelium after treatment with a carcinogen or in a vitamin A-deficient condition. Given the known growth-enhancing properties of TGF-α, our findings raise the possibility that elevated expression of TGF-α or an EGF-like peptide may function in an autocrine/paracrine manner to precede or induce the hyperplastic events observed in airway epithelium following vitamin A deficiency. Experiments are currently underway to identify such a mechanism and the cell type responsible for this regulatory mechanism in vivo.
ACKNOWLEDGMENTS

We thank Dr. Angie Rizzino for reviewing this paper prior to publication. Michael Trulson is thanked for his editing work, and the technical assistance from Jin Jie Hu and Chris Brown is greatly appreciated.

REFERENCES

Inhibition of Epidermal Growth Factor-like Growth Factor Secretion in Tracheobronchial Epithelial Cells by Vitamin A

Lisa A. Miller, Ling Zhong Cheng and Reen Wu


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/53/11/2527

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.