

Insulin-Like Growth Factor Binding Protein-6 Inhibits the Growth of Human Bronchial Epithelial Cells and Increases in Abundance with All-*trans*-Retinoic Acid Treatment

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Retinoids are potent inhibitors of human bronchial epithelial (HBE) cell growth. Retinoids initiate signaling through activation of nuclear receptors, but the signal transduction pathways that mediate growth inhibition have not been defined. In this study, we investigated the expression of insulin-like growth factor (IGF)-binding protein (IGFBP)-6 as a potential mediator of retinoid actions. IGFBP-6 is a secreted glycoprotein that inhibits the bioavailability of IGFs, which are potent mitogens of HBE cells. IGFBP-6 was detected by immunohistochemical staining in the basal epithelial layer of human bronchial organ cultures, and all-*trans*-retinoic acid (t-RA) treatment increased the intensity of IGFBP-6 immunostaining. In primary cultures of HBE cells treated with t-RA, IGFBP-6 messenger RNA and protein levels increased within 6 and 24 h, respectively, and IGFBP-6 was detected in the conditioned media at 48 h. The effect of IGFBP-6 on HBE cell growth was investigated with a recombinant adenoviral vector, Ad5CMV-BP6, which expresses IGFBP-6 under the control of a cytomegalovirus promoter. IGFBP-6 overexpression induced a proliferative arrest of HBE cells with no evidence of apoptosis. These findings provide the first evidence that IGFBP-6 is expressed in the bronchial epithelium and that IGFBP-6 may contribute to the biologic effects of retinoids on HBE cells.

Retinoids, including retinol (vitamin A) and retinoic acid stereoisomers, are potent modulators of tracheobronchial epithelial cell growth and differentiation. Rodents deprived of retinol develop squamous metaplasia of the tracheobronchial epithelium, and normal epithelial differentiation is restored by retinol supplementation (1–3). In tissue culture, human bronchial epithelial (HBE) cells undergo squamous differentiation with a variety of agents, and all-*trans*-retinoic acid (t-RA) inhibits this process (4–9). HBE cells treated with t-RA in collagen gels or semi-permeable membranes develop mucociliary features (4, 9–11). Grown in monolayer cultures at low density, HBE cells treated with t-RA undergo growth arrest with no evi-

dence of morphologic differentiation (12). The diverse morphologies exhibited by HBE cells in response to retinoid treatment support the presence of a complex network of retinoid signaling pathways regulated by cell culture conditions.

Retinoids are ligands for nuclear receptors, including those belonging to the retinoic acid receptor (RAR) and retinoid X receptor (RXR) gene families (reviewed in Reference 13), which form RXR:RAR heterodimers and RXR homodimers. Retinoid receptor transcriptional activity is regulated by ligand binding. Of the known natural retinoids, t-RA activates RARs and 9-*cis*-retinoic acid activates both RARs and RXRs (13). Ligand binding causes changes in retinoid receptor conformation, inducing retinoid receptors to dissociate from transcriptional corepressors and associate with transcriptional coactivators (reviewed in Reference 14). Through these mechanisms, retinoid receptors directly activate the expression of genes that contain retinoid response elements (RAREs) in their gene promoters. Several studies have demonstrated the importance of retinoid receptors in mediating the biologic effects of retinoids (15, 16). We and other investigators have demonstrated that t-RA is a potent inhibitor of HBE cell growth, which occurs through activation of RXR:RAR heterodimers (17, 18), but the signaling pathways that mediate this growth inhibition have not been completely defined.

An important mediator of retinoid actions is the family of insulin-like growth factor (IGF) binding proteins (IGFBPs). There are six IGFBP family members (IGFBP 1 to 6) (reviewed in Reference 19). IGFBPs are secreted proteins that bind to IGFs I and II in the extracellular matrix with high affinity and specificity, reducing IGF bioavailability. With the exception of IGFBP-6, which binds to IGF-II with higher affinity than it does to IGF-I by two orders of magnitude, IGFBPs bind to IGF-I with greater affinity. A closely related family of genes termed IGFBP-related proteins (IGFBP-rP), including *mac25*, *CTGF*, *cyt61*, and *nov*, have recently been cloned that bind to IGFs with relatively low affinity and share the NH₂-terminal cysteine-rich region of IGFBPs 1 through 6 but have no homology to their COOH-terminal regions (20). Retinoids activate the expression of IGFBP-3 and -6, and IGFBP-rP1 in specific cell types, which contributes, in part, to the biologic effects of retinoids on these cells (21–25).

In the lung, IGF-I and -II are expressed and are potent mitogens of normal and neoplastic cells derived from the bronchial epithelium (26–29). Because IGFBP-6 is a crucial regulator of IGF bioavailability, we hypothesized that IGFBP-6 is expressed in HBE cells, increases in abun-

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Abbreviations: bromodeoxyuridine, BrdU; BrdU triphosphate, Br-dUTP; complementary DNA, cDNA; green fluorescent protein, GFP; human bronchial epithelial, HBE; insulin-like growth factor, IGF; IGF binding protein, IGFBP; multiplicity of infection, MOI; messenger RNA, mRNA; polymerase chain reaction, PCR; plaque-forming units, PFU; retinoic acid receptor, RAR; an adenovirus that expresses luciferase driven by a respiratory syncytial virus promoter, RSV-LUC; retinoid X receptor, RXR; transforming growth factor, TGF; all-*trans*-retinoic acid, t-RA; Western ligand blot, WLB.

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dance with retinoid treatment, and exerts growth-inhibitory effects on HBE cells. Here, we present evidence that IGFBP-6 is expressed in the epithelial layer of human bronchial organ cultures and in primary cultures of HBE cells, and that in both models t-RA treatment increased the abundance of IGFBP-6 protein. Infection of HBE cells with a recombinant adenovirus that expresses IGFBP-6 induced a proliferative arrest with no evidence of apoptosis. These findings provide the first evidence that IGFBP-6 is expressed in the bronchial epithelium and that IGFBP-6 may contribute to the biologic effects of retinoids on HBE cells.

Materials and Methods

Conditions for Primary Cultures of HBE Cells and Bronchial Organs

Primary cultures of HBE cells were grown from bronchial mucosal biopsy samples resected from fresh surgical specimens as previously described (30). Briefly, the mucosal layer was sterilely stripped from bronchial specimens, cut into small pieces, and placed on a plastic tissue-culture plate containing a thin layer of medium. When HBE cells had grown from these tissues into a 60% confluent monolayer population, they were expanded for use in experiments. HBE cells were grown on standard plasticware (Falcon; Becton Dickinson, Bedford, MA) in Keratinocyte Serum-Free Medium (GIBCO BRL, Gaithersburg, MD) containing epidermal growth factor (EGF) and bovine pituitary extract (BPE) at 37°C with a pCO₂ of 5%. Cells were treated with t-RA (Sigma Chemical Co., St. Louis, MO) in the absence of EGF and BPE. Recombinant IGF-II used in the treatment of HBE cells was purchased (GIBCO BRL).

Bronchial organ cultures were performed as previously described (31). Briefly, fresh bronchial tissue from lobectomy specimens was cut into full-thickness fragments, 1 mm in diameter, and placed in tissue-culture plates (12-mm wells) base-coated with 0.5 ml of 0.5% agar (Agar Noble; Difco Laboratories, Detroit, MI) in Minimal Essential Medium (GIBCO BRL) with 10% fetal bovine serum (GIBCO BRL). The agar was overlaid with 0.5 ml of media, which was changed twice weekly. The epithelial layer was shed in most specimens at the time of establishment in culture, leaving a denuded mucosal surface from which the epithelium regenerated over the ensuing 4 wk. Although we did observe some variability in timing, by Day 21 the epithelium has completely regenerated in most specimens. After epithelial regeneration, organ cultures were treated with 1 μM t-RA for 3 d.

Northern Blot Analysis

Total cellular RNA was isolated from cells by the acid guanidium/phenol/chloroform extraction method as previously described (30). A total of 20 μg total cellular RNA was electrophoresed on an agarose gel, blotted onto a nylon membrane (Duralon UV; Stratagene, Inc., La Jolla, CA) and hybridized to [³²P]-labeled IGFBP-6 complementary DNA (cDNA) as previously described (30). The human IGFBP-6 cDNA was a 359-base pair fragment amplified by polymerase chain reaction (PCR) from 1 μg cDNA, which was prepared from total cellular RNA isolated from HBE cells treated for 24 h with 1 μM t-RA. The primers used to generate the IGFBP-6 PCR product were: sense (5'-GTTGCAGAG-GAGAATCC-3') and antisense (5'-CTTCCATTGCCATCTGG-3'). PCR conditions were: 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min (30 cycles).

Immunoblot and Western Ligand Blot Analysis

Conditioned media samples were collected from HBE cells at the end of 24-h intervals (0–24, 24–48, 48–72, 72–96, and 96–120 h) of

treatment with t-RA or adenoviral infection. Media were changed after each 24-h collection during t-RA treatment. Aliquots of conditioned media, determined on the basis of equal cell numbers, were concentrated 1:10 with Centriprep 10 filters (Amicon, Inc., Beverly, MA). Whole-cell lysates were isolated from HBE cells using MEGA RIPA buffer (50 mM Tris-HCl, pH 7.4; 100 mM NaF; 0.5% Nonidet P-40; 200 mM NaCl; 5 mM ethylenediaminetetraacetic acid; 5 mM ethyleneglycol-*bis*-(β-aminoethyl ether)-*N,N'*-tetraacetic acid; 20 mM β-glycerophosphate; 1 mM benzamidine; 1 mM phenylmethylsulfonyl fluoride; 1 μg/ml leupeptin; 10 μg/ml aprotinin; and 200 μM Na₃VO₄). HBE cell lysates or conditioned media were electrophoresed using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a BA-S-83-reinforced nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). Membranes were incubated with a rabbit polyclonal antihuman IGFBP-6 antibody (Austral Biotechnologies, San Ramon, CA) or goat polyclonal antihuman actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunodetection was performed with the ECL kit (Amersham Corp., Arlington Heights, IL). For Western ligand blot (WLB), the membrane was incubated with a 2 × 10⁶ counts/min of [¹²⁵I]-labeled IGF-I or -II and exposed to film as previously described (32). Recombinant IGFBP-3 (Diagnostic Systems Laboratories, Webster, TX) was included in WLB as a positive control.

Immunohistochemical Analysis of IGFBP-6 in Bronchial Organ Cultures

After treatment for 3 d with 1 μM t-RA or media alone, tissue was imbedded in paraffin and sections were mounted on aminoalkylsilane-coated slides (Histology Control System, Glen Head, NY). Tissue sections were postfixed in methanol and blocked with 0.5% Triton X-100 and 2% nonfat dry milk. The slides were incubated overnight at 4°C with 1:100 dilution of rabbit polyclonal antihuman IGFBP-6 antibody (Austral Biotechnologies). The Vectastain Elite ABC kit and diaminobenzidine were used for immunodetection as previously described (33). Sections were then counterstained with hematoxylin and eosin (H&E).

Generation of Recombinant IGFBP-6 Adenovirus

A full-length human IGFBP-6 cDNA (a gift from Dr. Shunichi Shimasaki, University of California-San Diego, San Diego, CA) (34) was inserted 5' of the bovine growth hormone polyadenylation signal between *Hind*III and *Xho*I of the pDE1/cytomegalovirus (CMV) shuttle vector (35). The IGFBP-6-containing shuttle vector and the recombinant plasmid pJM17 were cotransfected into 293 cells by calcium phosphate precipitation. After the cytopathic effect was detected, plaques were screened for newly-generated IGFBP-6 recombinant adenovirus (Ad5CMV-BP6) by PCR analysis. The presence of wild-type IGFBP-6 cDNA in the recombinant adenovirus was confirmed by dideoxy-DNA sequencing. Viral titers were determined by plaque assays as previously described (36).

Bromodeoxyuridine Incorporation

HBE cells were infected with Ad5CMV-BP6, Ad5CMV, or RSV-LUC (an adenovirus that expresses luciferase driven by a respiratory syncytial virus promoter) at a multiplicity of infection (MOI) of 40 plaque-forming units (PFU)/cell. At 3 d later, cells were treated with 10 μg/ml bromodeoxyuridine (BrdU) (Sigma) for 2 h, trypsinized, and fixed onto slides as described previously (17). The cells were incubated with a monoclonal antibody to BrdU (mAbBR3; Caltag Laboratories, Inc., Burlingame, CA) for 45 min at 37°C. The Vectastain ABC kit and diaminobenzidine were used for the detection of BrdU incorporation. The cells were then counterstained with Giemsa. The percentages of cells incorporating BrdU were determined among 10³ randomly counted cells.

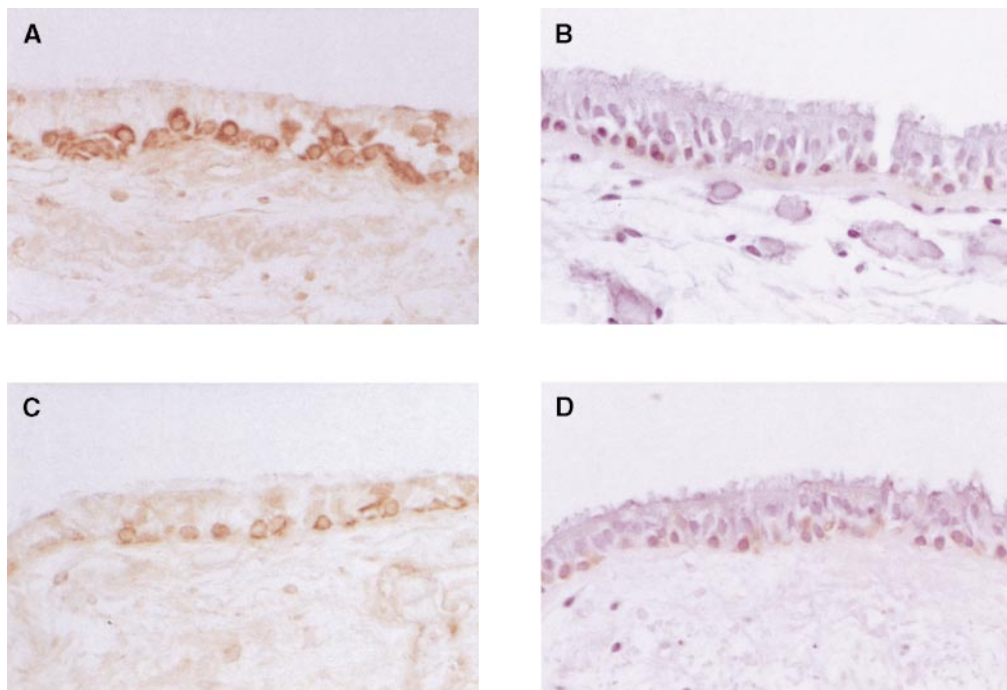


Figure 1. t-RA increased IGFBP-6 expression in the epithelium of bronchial organ cultures. Bronchial organ cultures were treated for 3 d with 1 μ M t-RA (A and B) or media alone (C and D) and then subjected to immunohistochemical staining for IGFBP-6. Immunostained sections were photographed after H&E counterstaining (B and D) or without counterstaining (A and C). Cells that stained positively for IGFBP-6 are brown. The illustrated tissue sections are representative of other sections within the specimens.

Assay for Induction of Apoptosis

HBE cells were infected with Ad5CMV or Ad5CMV-BP6 (MOI 40 PFU/cell) for 48 h and then examined by *in situ* end-labeling to quantitate the number of apoptotic cells. Apoptosis was measured using the APO-BRDU staining kit (Phoenix Flow Systems, San Diego, CA). Briefly, both floating and adherent cells were collected and fixed by 1% paraformaldehyde followed by 70% ethanol as described by the manufacturers. DNA breaks were detected by terminal deoxynucleotidyl transferase (TdT)-induced incorporation of BrdU triphosphate (Br-dUTP) into the 3'-OH ends of DNA strands. Cells were analyzed by a FACSCAN flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488-nm argon laser and CellQuest software. A dual display of DNA area (linear red fluorescence) and Br-dUTP incorporation (FITC-PRB-1) was used to determine the percentage of apoptotic cells in the population.

Results

IGFBP-6 Expression Increases with t-RA Treatment in Human Bronchial Epithelium

Because IGFBP-6 is a secreted protein and acts through autocrine and paracrine mechanisms, its expression should be examined *in situ* to identify its cell of origin and the surrounding tissue it might affect. For these studies, human bronchial organ cultures were established using bronchial mucosa resected from lobectomy specimens (Figure 1). Histologic examination of these organ cultures revealed areas of normal respiratory mucosa, with a basal proliferative region underneath surface columnar cells that were ciliated, contained mucous, and exhibited nuclear polarization with cytoplasm on the luminal aspect of the cell. These areas contained normal submucosal glands and goblet cells.

IGFBP-6 expression was examined by immunohistochemical staining. IGFBP-6 was visible in the cytoplasm of cells in

the epithelial layer of untreated bronchial organ cultures (Figures 1C and 1D). We investigated the effect of t-RA treatment on IGFBP-6 expression. Compared with the effects of media alone, treatment for 3 d with 1 μ M t-RA increased IGFBP-6 staining intensity in epithelial cells of bronchial organ cultures (Figures 1A and 1B).

t-RA Treatment Increased IGFBP-6 Expression in Primary Cultures of HBE Cells

We further investigated IGFBP-6 expression in primary cultures of epithelial cells grown from bronchial mucosal biopsies. The effect of t-RA on IGFBP-6 expression was investigated at the messenger RNA (mRNA) and protein levels. Northern analysis revealed IGFBP-6 mRNA in untreated cells, and t-RA increased IGFBP-6 expression in a

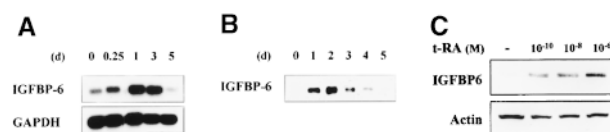


Figure 2. t-RA increased IGFBP-6 expression in primary cultures of HBE cells. IGFBP-6 Northern analysis (20 μ g total cellular RNA/lane) (A) and IGFBP-6 immunoblot analysis (B and C) were performed on conditioned media (20 μ l/lane) (B) and whole-cell lysates (50 μ g/lane) (C) prepared from HBE cells. The Northern membrane was hybridized to glyceraldehyde-3-phosphate dehydrogenase to examine the relative amounts of RNA loaded per lane. Time-dependent effects were examined by treating cells for the indicated number of days (d) with 1 μ M t-RA or media alone (lane 0) (B). Dose-dependent effects of t-RA on IGFBP-6 were examined by treating cells with the indicated doses of t-RA for 72 h (C). The membrane was immunoblotted with antiactin antibody to examine relative amounts of cell lysate loaded per lane.

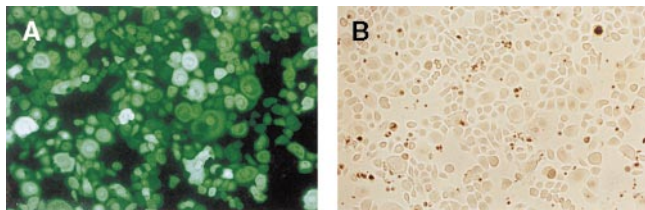


Figure 3. Gene transfer efficiency of Ad5CMV-GFP in HBE cells. HBE cells were infected with Ad5CMV-GFP at a MOI of 40 PFU/cell for 3 d and examined by fluorescence (A) and phase contrast (B) microscopy. Infected cells appear *green* under fluorescence microscopy.

time-dependent manner (Figure 2A). IGFBP-6 mRNA levels peaked at 24 h and returned to basal levels by 5 d. Secretion of IGFBP-6 protein was examined by immunoblot analysis of conditioned media collected from HBE cells after 24-h intervals of treatment with 1 μ M t-RA. Media were changed daily so that each sample collection represented only a 24-h time period. IGFBP-6 was detectable in media from t-RA-treated cells within 24 h, peaked at 48 h, and was undetectable at 5 d (Figure 2B). Treatment with t-RA increased intracellular IGFBP-6 protein levels in a dose-dependent fashion (Figure 2C).

IGFBP-6 Overexpression Inhibited HBE Cell Growth

We investigated the growth-regulatory effects of IGFBP-6 when overexpressed with a recombinant adenoviral vector under the control of the human CMV promoter (Ad5CMV). Infection efficiency of Ad5CMV-based vectors in primary cultures of HBE cells was high (> 90%), as shown by infection with Ad5CMV-GFP, which expresses green fluorescent protein (GFP) (Figure 3). We examined the levels of IGFBP-6 expressed intracellularly and secreted from HBE cells infected with Ad5CMV-BP6 or the parental vector Ad5CMV as a negative control. Immunoblot analysis revealed increased levels of intracellular (Figures 4A and 5A) and secreted (Figures 4B and 5B) IGFBP-6 after infection with Ad5CMV-BP6. IGFBP-6 expression increased in a viral titer-dependent (Figure 4) and time-dependent (Figure 5) manner. IGFBP-6 immunoreactive species of different molecular weights were observed that could represent proteolytic fragments or glycosylated forms of IGFBP-6. The functional state of adenoviral IGFBP-6 was investigated by examining IGF binding activity of conditioned media from HBE cells infected with Ad5CMV-BP6 or Ad5CMV. WLB was performed using [¹²⁵I]-IGF-I or [¹²⁵I]-IGF-II (Figure 6), which demonstrated increased IGF-II

binding activity in cells infected with Ad5CMV-BP6 but not Ad5CMV. Minimal IGF-I binding activity was detected, indicating that adenoviral IGFBP-6 specifically bound IGF-II.

The effect of Ad5CMV-BP6 infection on cell proliferation was examined. The percentage of cells actively cycling was determined by *in situ* analysis of BrdU incorporation after 3 d of infection (MOI 40 PFU/cell) with Ad5CMV-BP6, Ad5CMV, or RSV-LUC as an additional control. The experiment was performed three times using HBE cells derived from different biopsies, and the mean percentage (\pm standard deviation [SD]) of cells that incorporated BrdU was determined. A prominent reduction in BrdU incorporation was observed in cells infected with Ad5CMV-BP6 relative to the effects of Ad5CMV, RSV-LUC, or treatment with media alone (Figure 7). These findings provide evidence that IGFBP-6 overexpression was growth-inhibitory in HBE cells.

Because IGFBP-3 is a potent inducer of programmed cell death (36), we investigated whether IGFBP-6 inhibits HBE cell growth through a similar mechanism. TdT-induced incorporation of Br-dUTP was measured by flow cytometric analysis of HBE cells 48 h after infection with Ad5CMV or Ad5CMV-BP6 (MOI 40 PFU/cell). HL-60 cells treated with cytosine arabinoside were used as a positive control. In three different experiments, Br-dUTP incorporation was undetectable in cells infected with either adenovirus (data not shown). Consistent with these findings, HBE cells were > 90% viable by trypan blue exclusion 48 h after infection with these adenoviruses. These findings suggest that IGFBP-6 overexpression induced a proliferative arrest in HBE cells with no evidence of programmed cell death.

Discussion

Retinoids are known to regulate the growth and differentiation of HBE cells, but the signal transduction pathways that mediate retinoid actions on HBE cells have not been completely defined. Because IGFs are expressed in the lung and are potent mitogens of normal and neoplastic cells derived from the bronchial epithelium, we investigated the expression of IGFBP-6, a crucial regulator of IGF bioavailability. We found that IGFBP-6 was expressed in the basal epithelial cells of bronchial organ cultures and in primary cultures of HBE cells, increased in abundance with retinoid treatment, and exerted potent growth inhibitory effects on HBE cells.

Treatment of primary cultures of HBE cells with t-RA increased the mRNA and protein levels of IGFBP-6. The

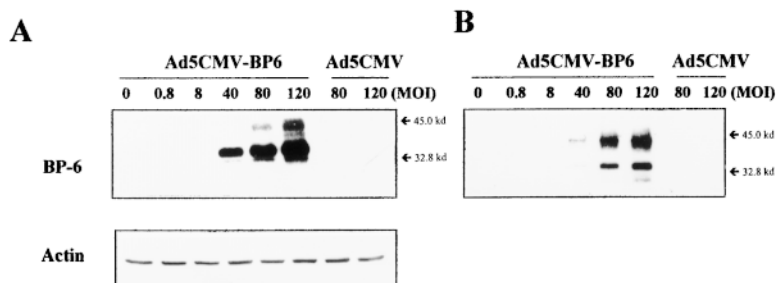


Figure 4. Viral titer-dependent expression of IGFBP-6 in HBE cells infected with Ad5CMV-BP6. Immunoblot analysis was performed to examine IGFBP-6 expression in whole-cell lysates (40 μ g/lane) (A) or conditioned media (20 μ l/lane) (B) prepared from HBE cells infected for 3 d with Ad5CMV or Ad5CMV-BP6 at MOIs of 0 to 120 PFU/cell. The relative amounts of whole-cell lysate loaded per lane were determined by immunoblotting to actin.

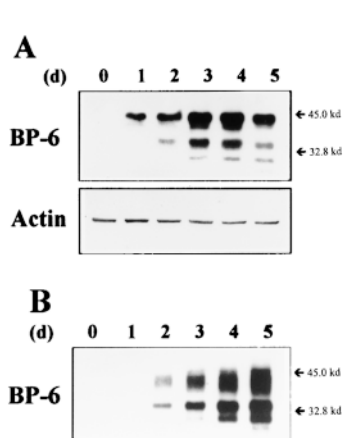


Figure 5. Time-dependent expression of IGFBP-6 in HBE cells infected with Ad5CMV-BP6. Immunoblot analysis of IGFBP-6 was performed on whole-cell lysates (40 µg/lane) (A) or conditioned media (20 µl/lane) (B) prepared the indicated number of days (d) after infection of HBE cells with Ad5CMV-BP6 (MOI of 40 PFU/cell). The relative amounts of whole-cell lysate loaded per lane were determined by immunoblotting to actin.

increase in IGFBP-6 mRNA occurred within the first 24 h and was transient, returning to basal levels by 5 d. Retinoids increase IGFBP-6 mRNA and protein levels in other cell types, including osteoblasts, fibroblasts, breast cancer, and neuroblastoma cells (21–25). IGFBP-6 mRNA levels have been shown to increase after retinoid treatment through transcriptional and post-transcriptional mechanisms (23). Whether retinoids transcriptionally activate IGFBP-6 expression directly through RAREs in the IGFBP-6 gene promoter or indirectly through other regulatory elements has not been determined. Exploring this question will require identifying the important regulatory elements in the human IGFBP-6 promoter, which have not yet been reported.

We found that primary cultures of HBE cells were efficiently infected with a recombinant adenovirus expressing GFP. The efficiency with which HBE cells are infected *in vitro* by recombinant adenoviruses varies by report (37, 38). Our findings indicate that Ad5CMV-BP6 infection induced high IGFBP-6 expression levels and enhanced binding activity specifically to IGF-II, which indicate that adenoviral IGFBP-6 is functional and support the use of adenoviral vectors in examining the biologic role of specific gene products in HBE cells. The multiple forms of IGFBP-6 observed in HBE cells suggest that adenoviral

IGFBP-6 was either post-translationally modified or cleaved. IGFBP-6 is known to be O-glycosylated and cleaved, which regulates IGFBP-6 activity (39).

IGFBP-6 overexpression in primary cultures of HBE cells induced a proliferative arrest with no evidence of programmed cell death. The growth-regulatory effects of IGFBPs have been examined in other cell types. Prostate cancer cells undergo programmed cell death after treatment with recombinant IGFBP-3 or stable transfection with IGFBP-3 expression plasmids (40). IGFBP-5 expression increases in breast cancer cells treated with the antiestrogen ICI 182780, and antisense IGFBP-5 abrogates the growth-inhibitory effect of this compound (41). Neuroblastoma cells stably transfected with an IGFBP-6 expression plasmid undergo proliferative arrest with no evidence of apoptosis (42). Thus, in these studies, IGFBP-3 and -6 caused distinct biologic effects; IGFBP-3 induced programmed cell death, and IGFBP-6 induced proliferative arrest. There are several factors that could have contributed to the different findings in these studies. First, the cells in these studies originated from different tissue types and had different transformation states. Second, different methods were used to overexpress IGFBPs (adenoviral versus plasmid vectors), which could affect IGFBP expression levels and post-translational processing. Third, IGFBP-3 and -6 may act through distinct mechanisms. IGFBP-3 is known to activate both IGF-dependent and -independent pathways, but a role for IGF-independent mechanisms has not been demonstrated for IGFBP-6 (19). To address this question, we examined the effect of conditioned media from HBE cells infected with Ad5CMV-BP6 on the growth of HBE cells in the presence and absence of exogenous IGF-II. This experiment revealed that conditioned media inhibited growth to a similar extent in the presence and absence of IGF-II (data not shown). This finding is consistent with the hypothesis that IGFBP-6 mediates its effect through IGF-independent mechanisms, but to address this question definitively genetic approaches are needed, such as examining the growth inhibitory effects of Ad5CMV-BP6 on IGF-I receptor-null HBE cells.

Our observation that IGFBP-6 increases in response to retinoid treatment and inhibits the growth of HBE cells is consistent with the conclusion that IGFBP-6 plays a role in the growth inhibitory effects of retinoids on HBE cells.

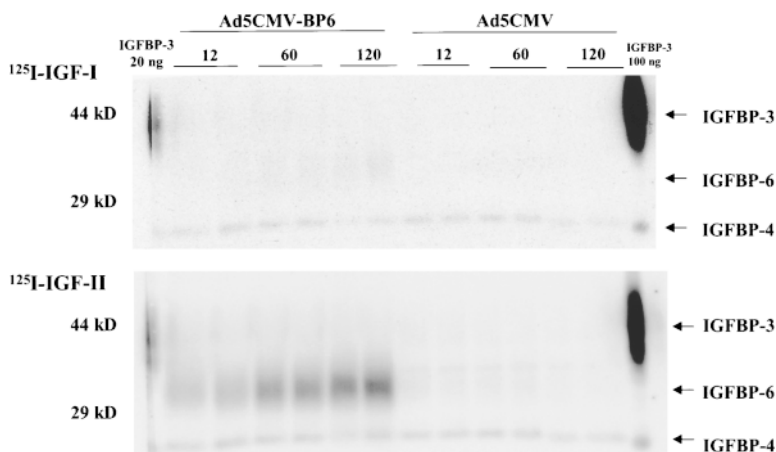


Figure 6. IGF-II binding activity is increased in HBE cells infected with Ad5CMV-BP6. Western ligand blotting was performed on conditioned media samples collected 72 h after infection of HBE cells with the indicated titers (MOI) of Ad5CMV-BP6 or Ad5CMV, using [¹²⁵I]IGF-I or [¹²⁵I]IGF-II as a probe. Recombinant IGFBP-3 was included on gels as a positive control. The positions of specific IGFBP family members are indicated on the *right* and molecular weight markers are indicated on the *left*. IGFBP-4 was also detected at basal levels in these conditioned media samples.

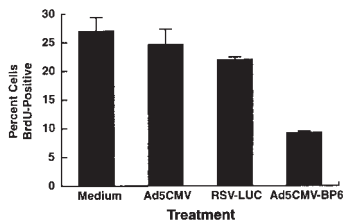


Figure 7. IGFBP-6 overexpression inhibited HBE cell growth. HBE cells were infected with MOI of 40 PFU/cell of the indicated adenoviral vectors. At 3 d later, the cells were treated with BrdU, trypsinized, cytospun onto slides, and

examined for BrdU incorporation by immunohistochemical staining. The percentages of cells that incorporated BrdU were calculated. The experiment was performed three times, and the results are expressed as the means (\pm SD) of the three experiments.

Supporting this hypothesis, the increase in IGFBP-6 expression correlates temporally with the growth inhibition induced by t-RA, and, like IGFBP-6, t-RA induces a proliferative arrest in approximately 60% of the cell population (17, 43). However, the role of IGFBP-6 in retinoid actions must be evaluated with respect to the other signaling pathways that are regulated in response to retinoid treatment. The activity of *c-jun* N-terminal kinase activity is inhibited by t-RA in HBE cells through activation of a mitogen-activated protein phosphatase (44, 45). Transglutaminase type II expression increases in rabbit tracheobronchial epithelial cells treated with a synthetic RAR-selective retinoid and contributes to the programmed cell death induced by this compound (18). Transforming growth factor (TGF)- β 2 increases in abundance in HBE cells treated with t-RA, is a potent inhibitor of HBE cell growth, and, under certain conditions, induces terminal squamous differentiation (6, 46). IGFBP-3 has been implicated in the growth inhibitory effects of retinoids, and, in breast cancer cells, retinoids increase IGFBP-3 expression through activation of a TGF- β signaling pathway (22). In HBE cells, however, blocking the biochemical effects of extracellular TGF- β does not alter the increase in IGFBP-3 induced by t-RA, suggesting that the increase in IGFBP-3 expression does not require activation of the TGF- β pathway through autocrine or paracrine mechanisms (46). These studies demonstrate the complexity of retinoid signaling pathways in HBE cells. Understanding how these pathways act alone and in combination to induce the diverse biologic effects of retinoids on the human bronchial epithelium will require additional study.

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