Effects of Insulin-like Growth Factor Binding Protein-3 and Farnesyltransferase Inhibitor SCH66336 on Akt Expression and Apoptosis in Non–Small-Cell Lung Cancer Cells


Background: Overexpression of insulin-like growth factor binding protein-3 (IGFBP-3) induces apoptosis in non–small-cell lung cancer (NSCLC) cells in vitro and in vivo. However, Ras-mediated signaling pathways could develop resistance to apoptotic activities of IGFBP-3 in NSCLC cells. We thus evaluated the therapeutic potential of the combination of IGFBP-3 and SCH66336, a farnesyltransferase inhibitor that blocks Ras activation, in NSCLC cell lines.

Methods: The effects of the combination of adenoviral IGFBP-3 and SCH66336 on proliferation and apoptosis of NSCLC cell lines (H1299, H596, A549, H460, H358, H322, and H226B) were assessed in vitro and in vivo by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a flow cytometry–based terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling assay, western blot analyses, and an NSCLC xenograft tumor model. The specific effects of Ad-IGFBP 3 and SCH66336 on mitogen-activated protein kinase and Akt were assessed by using adenoviral vectors that express constitutively active MEK1 or constitutively active Akt. Synergy was assessed by median effect analysis. Results: The combination of Ad-IGFBP3 and SCH66336 had synergistic antiproliferative effects in five cell lines (H1299, H596, A549, H460, and H322). Antiproliferative effects were accompanied by increased apoptosis in H460 cells in vitro. Overexpression of a constitutively active Akt but not a constitutively active MEK1 rescued H460 cells from apoptosis induced by single or combined treatment of Ad-IGFBP3 and SCH66336. In H1299 tumor xenografts, Ad-IGFBP3 and SCH66336 was associated with decreased tumor volume, increased apoptosis, and decreased Akt levels. Conclusions: The combination of Ad-IGFBP3 and SCH66336 decreased Akt expression and increased apoptosis in NSCLC cells in vitro and in vivo. Simultaneous treatment with IGFBP-3 and SCH66336 may have the potential to be an effective therapeutic strategy in NSCLC. [J Natl Cancer Inst 2004;96:1536–48]

Affiliations of authors: Department of Thoracic/Head and Neck Medical Oncology (HYL, YSC, KHC, KH, RL, WKH), Department of Thoracic Surgery (LJ), University of Texas M. D. Anderson Cancer Center, Houston, TX; Division of Biometry and Risk Assessment, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR (HM); Department of Hematology and Medical Oncology, Translational Oncology, Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA (FRK).

Correspondence to: Ho-Young Lee, PhD, Unit 432, Department of Thoracic/Head and Neck Medical Oncology, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030 (e-mail: hlee@mdanderson.org).

See “Notes” following “References.”

DOI: 10.1093/jnci/djh286
Journal of the National Cancer Institute, Vol. 96, No. 20, © Oxford University Press 2004, all rights reserved.
single chemotherapeutic agent or in combination with chemotherapy or irradiation to increase the efficacy of standard therapies. Moreover, lung cancer cells have higher levels of IGFs and IGF-binding sites than normal lung cells and respond mitogenically to exogenous IGFs (7,8), indicating that IGF-directed treatments might preferentially localize to lung tumors.

We previously found that expression of IGFBP-3 in the treatment of NSCLC, which is believed to be mediated by oncogenic Ras (17). Ras-mediated signaling pathways can be activated by multiple events, including the overexpression of IGFBP-3 and the risk of lung cancer (12,13). IGFBP-3 in the serum sequesters IGFs, reducing IGF availability to various cells and tissues. IGFBP-3 also has IGF-independent antiproliferative and pro-apoptotic effects. For example, IGFBP-3 overexpression inhibits the growth of IGF-1R–null fibroblasts (14). The effects of IGFBP-3 are probably mediated by other cell surface receptors, such as the type V transforming growth factor β (TGF-β) receptor (15). Recently, antitumor effects of recombinant human IGFBP-3 were observed in solid tumor models, including a lung carcinoma model (16). Thus, these findings provide a strong rationale for the use of IGFBP-3 as a therapeutic agent for the treatment of lung cancer.

One potential obstacle to using IGFBP-3 in the treatment of NSCLC, however, is the development of nonresponsiveness to IGFBP-3, which is believed to be mediated by oncogenic Ras (17). Ras-mediated signaling pathways can be activated by multiple events, including the overexpression of the epidermal growth factor receptor family and one of its ligands, TGF-β; mutations in Ras; reduced expression of PTEN; and amplification of a region of chromosome 3q that includes the p110 catalytic subunit of phosphoinositide 3-kinase (PI3K), all of which have been found in 24%–80% of lung tumor tissues from patients with NSCLC (18–24). Because Ras-mediated signaling pathways are numerous and complex, blocking Ras before it localizes to the cell membrane may be an attractive and practical target. Indeed, inhibitors of farnesyltransferase, an enzyme required for posttranslational processing and membrane translocation of Ras, have recently been introduced into clinical trials (25). Combinations of the farnesyltransferase inhibitor (FTI) SCH66336, one of the first such inhibitors to undergo clinical testing, with cyclophosphamide, 5-fluorouracil, vincristine, cisplatin, taxanes, or p53 gene therapy, have been reported to enhance antitumor activity in preclinical cancer models (25–29). In this study, we hypothesized that Ras-mediated signaling is critical to blocking IGFBP-3 activity and that blocking Ras-mediated signaling would increase the antitumor activity of IGFBP-3 in NSCLC cells. We tested this hypothesis by targeting Ras activation with SCH66336 and evaluated the consequence of treating NSCLC cells with the combination of SCH66336 and IGFBP-3.

METHODS

Reagents

Tissue culture reagents and plasticware were from Nunc (Roskilde, Denmark). An adeno viral vector expressing IGFBP-3 (9), referred to here as Ad-IGFBP3; an adeno viral vector expressing constitutively active MEK1 (Ser217/221 to Glu) (kindly provided by Dr. J. D. Molckentin, Cincinnati, OH) (30), referred to here as Ad-MEK1; an adeno viral vector expressing constitutively active Akt (MyrAkt) (31), referred to here as Ad-MyrAkt; and an adeno viral vector expressing empty vector, referred to here as Ad-EV, were amplified as described previously (17). IGF and epithelial growth factor (EGF) were purchased from R & D Systems (Minneapolis, MN). SCH66336 (+45-12-4-[8-chloro-3, 0-dibromo-6, 11-dihydro-5 benzocyclohepta (1,2-b) pyridin-11-y1]-1-piperidinyl]-2-o xoethyl]-1-piperidin carboxamide) was obtained from Schering-Plough (Kenilworth, NJ). We confirmed the activity of SCH66336 in in- farnesylation (32), measured as an enhancement in the level of unfarnesylated H-Ras in NSCLC cell lines (data not shown). LY294002, an inhibitor of PI3K, and PD98059, an inhibitor of mitogen-activated protein kinase MEK1, were purchased from Calbiochem-Novabiochem (Alexandria, New South Wales, Australia). Inhibitors were prepared as 20 mM stock solutions in dimethyl sulfoxide (DMSO) and stored at −20 °C.

Expression and Purification of TAT Fusion Protein

Proteins generated from the vector pTAT-HA have an N-terminal six-histidine leader sequence, which facilitates purification on nickel-based Sepharose columns; an 11-amino-acid transduction domain of the human immunodeficiency virus type 1 TAT protein, which facilitates the intracellular delivery of mature proteins; and a nine–amino-acid hemagglutinin tag (33). The full-length human IGFBP-3 coding region was inserted into the EcoRI–XhoI restriction site of the pTAT-HA bacterial expression vector (kindly provided by Dr. S. F. Dowdy, St. Louis, MO) to produce in-frame TAT fusion proteins (TAT-BP3).

IGFBP-3 primers used in this study are listed as follows (restriction enzyme sites incorporated to facilitate cloning are underlined): pTAT-BP3 (sense) 5′-ACGGCTCGAGGGCCG GAGCTCGGGGGGC-3′ (XhoI), (antisense) 5′-ACCGAATT CCTACTTGCTGTCACTGTTGAG-3′ (EcoRI). The presence of IGFBP-3 in pTAT-BP3 vector was confirmed by dideoxy-DNA sequencing. pTAT-BP3 vectors were expressed in Escherichia coli strain BL21 (DE3) pLYSs (Novagen, Madison, WI), according to the manufacturer’s recommended protocol. To purify the fusion protein, we lysed the bacterial cells by sonication in a buffer consisting of 20 mM phosphate buffer (pH 7.8), 500 mM NaCl, 100 mg of lysozyme, and protease inhibitors (0.1M phenylmethylsulfonyl fluoride, 0.5 mg/mL leupeptin, 1 mg/mL pepstatin, and 1 mg/mL apro pronin). E. coli lysates were denatured in 8 M urea, and IGFBP-3 proteins were purified by metal-affinity chromatography using Ni-NTA matrix (Qiagen, Chatsworth, CA) and gel filtration on a Sephadex G-25M (Amersham Pharmacia Biotech, Arlington Heights, IL) column, as previously described (33). Proteins were eluted from the Ni-NTA matrix by the addition of 500 mM imidazole and dialyzed against 1× phosphate-buffered saline (PBS) overnight at 4 °C.

The induction of IGFBP-3 expression in E. coli from pTATBP3 was examined by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis of the purified proteins through a 12% gel and Coomassie blue staining, and by western blot analysis for IGFBP-3. The binding of E. coli–expressed IGFBP-3 proteins to IGF-I was confirmed by western blot analysis (data not shown).
**Cells and Cell Culture**

Normal human bronchial epithelial (NHBE) cells were purchased from Clonetech (Palo Alto, CA) and maintained in keratinocyte serum-free medium (Life Technologies, Gaithersburg, MD) containing EGF (2 μg/mL) and bovine pituitary extract (25 μg/mL) (31). Ten human NSCLC cell lines with high endogenous levels of IGFBP-3 (H596, A549, and H460), detectable levels of IGFBP-3 (H358 and H226B), and undetectable levels of IGFBP-3 (H1299, H441, H322, H226B, and Calu-6) of IGFBP-3 (34) were used. Human NSCLC cell lines H1299, H226B, H596, A549, H460, and H358 were purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (GIBCO-BRL, Gaithersburg, MD) in a humidified atmosphere with 5% CO2.

To determine the effects of IGFBP-3 or SCH66336 on NSCLC cell proliferation, the NSCLC cell lines were plated at concentrations of 1 × 10^5 to 2 × 10^5 cells per well in 96-well plates. The next day, cells were mock-infected or infected with various titers of Ad-IGFBP3 or Ad-EV for 2 hours and then incubated in complete medium for the assay period. To test antiproliferative effects of SCH66336, we treated cells with either 0.1% DMSO or various concentrations of SCH66336 (final DMSO concentration = 0.1%) in complete medium, which was changed daily during the assay period. At the end of the assay period, the proliferation of treated cells was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described (31). Six replicate wells were used for each assay; data from replicate wells are presented as mean values with 95% confidence intervals (CIs). Three independent experiments were performed with similar results; representative results of one experiment are presented.

To test the effects of combined treatment with IGFBP-3 and SCH66336 or LY294002 on H460 cell proliferation, we infected cells with various titers of Ad-IGFBP3 or Ad-EV for 2 hours and then incubated in complete medium for the assay period. To test antiproliferative effects of SCH66336, we treated cells with either 0.1% DMSO or various concentrations of SCH66336 (final DMSO concentration = 0.1%) in complete medium, which was changed daily during the assay period. At the end of the assay period, the proliferation of treated cells was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described (31). Six replicate wells were used for each assay; data from replicate wells are presented as mean values with 95% confidence intervals (CIs). Three independent experiments were performed with similar results; representative results of one experiment are presented.

Western Blot Analysis

Whole-cell, nuclear, and cytosolic extracts from 10^6 cells were prepared in lysis buffer as described previously (31,34). Equivalent amounts of protein were resolved by SDS–polyacrylamide gels (7.5%–12%) and transferred to nitrocellulose membranes. After the membranes were blocked in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) and 5% (wt/vol) nonfat powdered milk, the membranes were incubated with primary antibodies diluted in TBS–5% nonfat milk at 4°C for 16 hours. Membranes were then washed multiple times with TBST and incubated with the appropriate horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature. The protein–antibody complexes were detected by using the enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL), according to the manufacturer’s recommended protocol. Representative results from two separate experiments are shown.
The following antibodies and working dilutions, suggested by the manufacturers, were used for the western blots: rabbit polyclonal anti–caspase-3 (1:2000) and rabbit polyclonal anti–Bax antibodies (1:2000) (Pharmingen, San Diego, CA); rabbit polyclonal anti–Bcl-2 (1:1000), rabbit polyclonal anti–Bel-xL, and rabbit polyclonal anti–poly(ADP-ribose) polymerase (PARP) antibody (1:1000) (VIC 5; Roche Molecular Biochemicals, Indianapolis, IN); mouse monoclonal antiphosphorylated MAPK (ppP44/42 MAPK) (Thr202/Tyr204) (1:500), rabbit polyclonal antibodies against phosphorylated JNK (pJNK) (1:1000), phosphorylated p38 MAPK (ppp38 MAPK) (1:1000), p38 MAPK (1:1000), phosphorylated Akt (pAkt) (Ser473) (1:1000), phosphorylated glycogen synthase kinase 3 (pGSK-3β) (Ser21/9) (1:1000), phosphorylated phospho-IGF-1R (Tyr131) (1:1000), or IGF-1Rα (1:1000) (Cell Signaling Technology, Beverly, MA); rabbit polyclonal anti–GSK-3β antibody (1:1000) (BD Transduction Laboratories, Lexington, KY); goat polyclonal antibodies against extracellular related kinase 1 (p44 MAPK) (1:1000) or JNK (1:1000), rabbit polyclonal anti–Akt1 and anti–β-actin (1:4000) antibodies (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti–mouse immunoglobulin G (IgG)–horseradish peroxidase conjugate (1:2000) (DAKO, Carpinteria, CA); and donkey anti–rabbit IgG–horse radish peroxidase conjugate (1:2000) and rabbit anti–goat IgG–horseradish peroxidase conjugate (1:2000) (Amersham Pharmacia Biotech).

Apoptosis Analysis

H460 cells were plated at a concentration of 2 × 10^5 cells/well in six-well plates. The next day, cells were mock-infected or infected with Ad-EV or Ad-IGFBP3 for 2 hours and then treated with SCH66336 (final DMSO concentration = 0.1%) or DMSO (0.1%) for 3 days. Apoptosis was assessed using a flow cytometry–based modified terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labeling (TUNEL) assay. For TUNEL analysis, both adherent and nonadherent cells were harvested and pooled, fixed with 1% paraformaldehyde and 70% ethanol, and processed using the APO-BrdU staining kit (Phoenix Flow Systems, San Diego, CA), as described previously (31). Cells treated with DMSO were used to gate the control nonapoptotic populations, and cells treated with deguelin (31) were used as a positive control. An internal control (HL-60 cells treated with camptothecin to induce apoptosis) provided in the apoptosis detection kit was also used to ensure that the TUNEL reaction occurred during the staining procedure. Two independent experiments were performed; representative results of one experiment are presented.

Metabolic Labeling and Immunoprecipitation

Metabolic labeling was performed with H460 cells (2 × 10^6 cells in 100-mm^2 plates) that were infected first with Ad-EV (25 pfu/cell) or Ad-IGFBP3 (25 pfu/cell) for 2 hours and then treated with 0.1% DMSO or SCH66336 (1 μM) for 3 days. Cells were washed in PBS and incubated in RPMI medium without methionine and cysteine (Sigma, St. Louis, MO) for 1 hour. Trans. 35S label (0.5 mCi; ICN) was added to the culture medium, and the cells were incubated for 1 hour. After the pulse, the medium containing the label was removed and replaced with fresh medium containing methionine and cysteine added to final concentrations of 150 mg/L. After 3 hours, cells were washed in ice-cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer. Lysates containing equal amounts of protein (30 μg) were used in immunoprecipitation analysis using 1 μg of antibody detecting Akt (Santa Cruz Biotechnology) and 40 μL of a 50% slurry of Sepharose protein A beads (Pharmacia-LKB Biotechnology, Piscataway, NJ). The immunoprecipitates were washed five times with lysis buffer, separated by SDS–polyacrylamide gel electrophoresis, and analyzed by autoradiography, as described previously (37). A preimmune serum was used as a negative control for immunoprecipitation. Cell extracts were also subjected to Western blot analysis for β-actin to ensure that equal amounts of protein were used. Two independent experiments were performed with similar results; representative results of one experiment are presented.

In Vivo Tumor Model

The effect of the combination of IGFBP-3 and SCH66336 on established subcutaneous tumor nodules was studied in athymic nude mice (Harlan-Sprague-Dawley, Indianapolis, IN) in a defined pathogen-free environment. Briefly, 4-week-old female nude mice were irradiated with 350 rad from a cesium-137 source and then were injected subcutaneously with 10^7 H1299 cells in 100 μL of growth medium at a single dorsal site. The mice were randomly assigned to one of six treatment groups, with each group containing five mice. Group 1 received PBS and hydroxyl-propyl-beta-cyclodextrin (HPCD), a vehicle for SCH66336; group 2 received PBS and SCH66336; group 3 received Ad-EV and HPCD; group 4 received Ad-EV and SCH66336; group 5 received Ad-IGFBP3 and HPCD; and group 6 received Ad-IGFBP3 and SCH66336. After the tumor volume reached approximately 75 mm^3 (day 0), mice received a single intratumoral injection of 2 × 10^5 particles of Ad-IGFBP3 or Ad-EV in 100 μL of PBS or 100 μL of PBS (as a control). On days 2 through 10, mice received SCH66336 (40 mg/kg of body weight) or 20% HPCD (100 μL) delivered orally twice a day. Tumor size was measured every day for 16 days, when the mice in groups 1–4 were killed because of excessive tumor burden. Tumor growth was quantified by measuring the tumors in three dimensions with calipers. Mice with necrotic tumors or tumors 1.5 cm or greater in diameter were immediately killed. The results were expressed as the mean tumor volume (n = 5) with 95% confidence intervals. The statistical significance of differences in tumor growth between the combination treatment group and the single-agent treatment groups was analyzed using two-way analysis of variance.

In Vivo Apoptosis and Immunohistochemistry

To determine whether the combination of Ad-IGFBP3 and SCH66336 induced apoptosis in tumor nodules in vivo, we collected tumor tissues from each treatment group, fixed them with 10% formaldehyde, embedded them in paraffin, and sectioned them. For some tumors, sections (5-μm thick) were subjected to the TUNEL assay to detect apoptosis-induced DNA fragmentation, as previously described (38). For other tumors, sections were processed for immunohistochemistry and deparaffinized through a series of xylene baths and rehydrated through a series of graded ethanol baths. The sections were immersed in methanol containing 0.3% hydrogen peroxidase for 20 minutes to block endogenous peroxidase activity and then incubated in 2.5% blocking serum for 1 hour to reduce nonspecific antibody
binding. Sections were incubated overnight at 4 °C with primary antibody against Akt (diluted 1:100 in 2.5% blocking serum) or Bcl-xL (diluted 1:100). The sections were then processed using standard avidin–biotin immunohistochemical techniques according to the manufacturer’s recommendations (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen, and commercial hematoxylin was used as a counterstain. H1299 cells were stained at the same time to serve as a positive control. Akt and Bcl-xL labeling indices were defined as the percentage of tumor cells displaying membranous or cytoplasmic immunoreactivity, respectively, and were calculated by counting the number of stained tumor cells among more than 1000 tumor cells from multiple representative areas of each tissue section. In this study, we used a 5% labeling index as a cutoff point. We evaluated the slides based on five score levels. We chose a 5% labeling index as a cutoff point for statistically significant results (P<.05). On the basis of the immunohistochemical staining results, we considered tumors in which less than 5% of the cells stained positive to have decreased Akt and Bcl-xL expression. All slides were evaluated and scored independently by two pathologists who were blinded to the clinical information of the subjects.

Statistical Analysis

Cell survival comparisons among groups were analyzed by the two-way analysis of variance for 2 × 2 factorial design. All means from triplicate samples and 95% confidence intervals were calculated using SAS software (Release 8.02; SAS Institute, Cary, NC). In all statistical analyses, two-sided P values less than .05 were considered statistically significant.

RESULTS

Expression of IGF Pathway Components in NSCLC Cells

To investigate the role of the IGF pathway in NSCLC cells, we first examined the level of expression and the phosphorylation status of IGF-1R and its signaling component Akt in 10 NSCLC cell lines and in NHBE cells by western blot analysis (Fig. 1, A). The level of IGF-1R was associated with its level of phosphorylation; i.e., cell lines that expressed detectable levels of IGF-1R also expressed phosphorylated IGF-1R. Seven of 10 NSCLC cell lines expressed higher levels of IGF-1R and phosphorylated IGF-1R than NHBE cells, indicating that the IGF-1R pathway was activated in most of the NSCLC cell lines tested. Phosphorylated Akt levels were also higher in nine of 10 NSCLC cell lines than in NHBE cells, although Akt levels were similar among all cell lines. Only five of 10 NSCLC cell lines (H1299, H596, H460, H226B, and Calu6) expressed both phosphorylated Akt and phosphorylated IGF-1R.

To determine whether Akt phosphorylation was dependent on IGF-1R activation, levels of phosphorylated IGF-1R and phosphorylated Akt were assessed by western blot analysis in H1299 cells that had been incubated in serum-free medium for 24 hours and then stimulated with exogenous IGF-1 (Fig. 1, B). In the presence of IGF-1, levels of phosphorylated IGF-1R and phosphorylated Akt increased. Increased phosphorylated IGF-1R in response to IGF-1 was also observed in other NSCLC cell lines (data not shown). These findings suggest that Akt can be activated (i.e., phosphorylated) by IGF-1R–dependent and IGF-1R–independent mechanisms.

Response of NSCLC Cells to IGFBP-3 and SCH66336

The level of IGF-1R is associated with the mitogenic response to IGF-I (39). We found that inhibiting Akt and MAPK activation was associated with a decrease in IGF-1–induced IGF-1R phosphorylation in the NSCLC cells infected with Ad-IGFBP3 [Lee et al. (9) and data not shown]. Because activated Ras–mediated signaling could limit the therapeutic efficacy of IGFBP-3, we investigated the effects of single-agent Ad-IGFBP3 or cotreatment with SCH66336 on the proliferation of the NSCLC cell lines H1299, H596, A549, H460, H358, H226B, and H322. The IC50s, which are analogous to D50 for Ad-IGFBP3 and SCH66336 in these cell lines, ranged from 50 to 100 pfu/cell and 2 to 6 μM, respectively (data not shown). Growth was reduced by no more than 50% in cells infected with Ad-IGFBP3 or in cells treated with SCH66336 for 72 hours (Fig. 2, A). However, growth of cells infected with Ad-IGFBP3 and treated with SCH6636 was reduced by at least 75%. A combination effect in cells infected with Ad-IGFBP3 and treated with SCH6636 was statistically significant at a 5% level in H1299 (P<.001), H596 (P = .02), A549 (P = .006), H460 (P<.001), and H358 (P<.001) cells, suggesting that attenuation of Ras-mediated signaling augmented the antiproliferative effects of IGFBP-3 for these five NSCLC cell lines.

To investigate whether the antiproliferative effects of the combination of Ad-IGFBP3 and SCH6636 were synergistic, we analyzed the data from several fixed-dose ratios using the median effect method (36). As single agents, Ad-IGFBP3 and SCH6636 each inhibited H460 cell proliferation (Fig. 2, B-a, b). Growth inhibition by the combination of agents was greater than each agent alone at every ratio (Fig. 2, B-c). In H460 cells, the combination index was consistently less than 1 at every fixed ratio (Fig. 2, B-d), demonstrating synergistic growth-inhibitory effects of the combined treatment. Although the formula used to calculate the combination index of mutually nonexclusive drug interactions has recently been questioned (40), we determined...
Fig. 2. Effect of the combination of adenoviral vector containing insulin-like growth factor binding protein-3 (Ad-IGFBP3) and the farnesyltransferase inhibitor SCH66336 on the apoptosis of non–small-cell lung cancer (NSCLC) cells.

A) The NSCLC cell lines H1299, H596, A549, H460, H358, and H226B were seeded onto 96-well culture plates (1 × 10^3 to 2 × 10^3 cells/well) and allowed to adhere overnight. The next day, the cells were mock-infected (con) or infected with 25 plaque-forming units (pfu)/cell of Ad-EV (empty vector) or Ad-IGFBP3, before exposure to 0.1% dimethyl sulfoxide (DMSO) (–) or 1 μM SCH66336 (+). Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay after 3 days. Results are expressed as percent cell proliferation relative to the proliferation of mock-infected cells exposed to 0.1% DMSO. Each bar represents the mean value of six identical wells from a representative single experiment (n = 3). Error bars show the upper 95% confidence intervals. Open bars indicate mock-infected; striped bars = Ad-EV; solid bars = Ad-IGFBP3. **, (H1299, P < .001; H596, P = .02; A549, P = .006; H460, P < .001; and H358, P < .001, and H226B, P < .001) for the combination effect of cells treated with Ad-IGFBP3 and SCH66336. B) H460 cells were incubated for 72 hours with doses of SCH66336 (a) or Ad-IGFBP3 (b) alone (solid line) or combined (dotted line) at fixed ratios (1:0.2, 1:0.5, or 1:1). Growth of treated cells was plotted relative to survival of untreated cells. The maximum dose in mixtures is two times the 50% inhibitory concentration (IC50) for Ad-IGFBP3 or SCH66336. C) H460 cells mock-infected (con) or infected with 25 pfu/cell of Ad-EV (empty vector) or with Ad-IGFBP3 (5 or 25 pfu/cell) for 2 hours and then changed to the medium containing either 0.1% DMSO (–) or 1 μM SCH66336. After 72 hours, cells were processed for apoptosis by a flow cytometry–based modified terminal deoxynucleotidyl transferase (TdT)–mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) assay. DNA content was determined by uptake of propidium iodide (x-axis). Apoptotic cells were determined by the intensity of fluorescein isothiocyanate (FITC) staining (y-axis). Number of apoptotic cells is indicated by the number of FITC-positive cells of the total gated cells. Each value presented is the percentage of apoptotic cells. The percentage of dead cells was determined by flow cytometry analysis of propidium iodide–stained nuclei. Data shown are from a representative experiment of two experiments performed. D) Expression of caspase-3 proenzyme (32 kD), cleaved poly ADP-ribose polymerase (PARP) (86 kD), and Bcl family members (Bax, Bcl-2, and Bcl-xL) in H460 cells treated with Ad-IGFBP3 and SCH66336 (1 μM), alone or in combination as described above, were assessed by western blot analysis. Expression of β-actin was used as a loading comparison. Representative experiment of two experiments is shown.
the combination index of mutually nonexclusive drug interactions and found that Ad-IGFBP3 and SCH66336 had synergistic effects. Synergistic effects of the combined treatment were observed for H1299, H596, A549, H460, and H322 cells, but additive effects were seen for H226B and H358 cells (Table 1). Responses to Ad-IGFBP3 and SCH66336 were not associated with mutations in Ras or in p53, cell origin, or IGFBP-3 protein expression (41, 42).

Apoptotic Effects of IGFBP-3 and SCH66336 In Vitro

TUNEL staining and flow cytometry analysis were used to determine whether H460 cells infected with Ad-IGFBP3 and treated with SCH66336 underwent apoptosis. Approximately 1% of control H460 cells, 23% of H460 cells infected with Ad-IGFBP3, and 19% of H460 cells treated with SCH66336 underwent apoptosis (Fig. 2, C). By contrast, 83% of H460 cells infected with Ad-IGFBP3 and treated with SCH66336 underwent apoptosis.

Western blot analysis of apoptosis-related enzymes (caspase-3 and PARP) and proteins (Bcl-2, Bax, and Bcl-xL) showed that, after 72 hours, H460 cells infected with Ad-IGFBP3 and treated with SCH66336 had decreased levels of the inactive form of procaspase-3 (relative molecular mass = 32) and concomitant increased cleavage of the 113-kd poly ADP-ribose polymerase (PARP) to the 89-kd fragment (Fig. 2, D). Compared with levels in control cells, levels of Bcl-2 and Bcl-xL were lower in H460 cells infected with Ad-IGFBP3 and even lower or undetectable in H460 cells infected with Ad-IGFBP3 and treated with SCH66336. By contrast, levels of Bax were unchanged by either agent or the combination. These results suggest that the ratio of antiapoptotic proteins (i.e., Bcl-2, Bcl-xL) to proapoptotic proteins (i.e., Bax) is altered by IGFBP-3 and SCH66336 in NSCLC cells.

Effect of Ad-IGFBP3 and SCH66336 on MAPK and Akt Pathways in NSCLC Cells

We next investigated the mechanism by which Ad-IGFBP3 and SCH66336 increased apoptosis in H460 cells. FTIs inactivate Ras and can inhibit the in vitro growth of transformed cells with wild-type ras gene. Because the response of NSCLC cell lines to Ad-IGFBP3 and SCH66336 was not associated with mutations in the ras gene (Table 1), we determined the effects of Ad-IGFBP3 and SCH66336, both alone and in combination, on the downstream mediators of Ras signaling pathways, including p44/42 MAPK, stress-induced MAPK (JNK), p38 MAPK, and Akt.

Compared with levels of phosphorylated p44/42 MAPK, Akt, and GSK-3α/β (a downstream mediator of Akt) in vehicle-treated control cells, levels of these proteins were lower in H460 cells infected with Ad-IGFBP3 or treated with SCH66336, and even lower in H460 cells infected with Ad-IGFBP3 and treated with SCH66336 (Fig. 3, A). Although levels of phosphorylated JNK or p38 MAPK were slightly lower in cells infected with Ad-IGFBP3 than in control cells, the addition of SCH66336 to the infected cells had no consistent effect on the levels of these phosphorylated proteins. Each agent alone and their combination showed no effect on the basal levels of p44/42 MAPK, JNK, p38 MAPK, or GSK-3β. However, levels of Akt were decreased in H460 cells infected with Ad-IGFBP3 and treated with SCH66336.

We explored the mechanism that mediates the decrease in Akt expression levels by Ad-IGFBP3 and SCH66336 in H460 cells. Northern blot analysis revealed that levels of Akt mRNA were not affected by the treatment with Ad-IGFBP3 and SCH66336 (data not shown). With no evidence of a posttranscriptional regulation of Akt expression, we next investigated whether Akt expression was regulated posttranslationally by performing pulse–chase experiments. The stability of the Akt protein in cells infected with Ad-IGFBP3 and treated with SCH66336 was markedly decreased compared with protein stability in cells treated with Ad-IGFBP3 or SCH66336 alone, suggesting that combined treatment of Ad-IGFBP3 and SCH66336 decreased Akt protein levels posttranslationally (Fig. 3, B).

Effect of p44/42 MAPK and Akt on Apoptosis Induced by Ad-IGFBP3 and SCH66336

Because the combination of Ad-IGFBP3 and SCH66336 inhibited the activation of the p44/42 MAPK and PI3K/Akt pathways, we first tested whether the inhibition of these pathways with the MEK inhibitor PD98059 and the PI3K inhibitor LY294002, respectively, would increase the antiproliferative effects of Ad-IGFBP3 and SCH66336 on H460 cells. We used inhibitor doses that were sufficient to reduce the IGF- or EGF-stimulated phosphorylation of p44/42 MAPK or Akt (data not shown). The mean difference in proliferation of cells infected with Ad-IGFBP3 and treated with LY294002 (66.19%, 95% CI = 53.19% to 79.19%) was statistically significantly greater than the sum of the mean difference of cells infected with Ad-IGFBP3 alone (29.42%) (Fig. 4, A) (*P = .005). The mean difference in proliferation of cells treated with Ad-IGFBP3 and PD98059 was

Table 1. Non–small-cell lung cancer cell line characteristics and responsiveness to combination of adenoviral vector containing insulin-like growth factor binding protein 3 (Ad-IGFBP-3) and farnesyl transferase inhibitor SCH66336

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Histology</th>
<th>p53 status*</th>
<th>Ras mutation</th>
<th>IGFBP-3 expression†</th>
<th>Median effect analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1299</td>
<td>Large cell</td>
<td>Null</td>
<td>Mutant</td>
<td>–</td>
<td>Synergy</td>
</tr>
<tr>
<td>H596</td>
<td>Adenosquamous</td>
<td>Mutant</td>
<td>Wild-type</td>
<td>+</td>
<td>Synergy</td>
</tr>
<tr>
<td>A549</td>
<td>Adenocarcinoma</td>
<td>Wild-type</td>
<td>Mutant</td>
<td>+</td>
<td>Synergy</td>
</tr>
<tr>
<td>H460</td>
<td>Large cell</td>
<td>Wild-type</td>
<td>Mutant</td>
<td>+</td>
<td>Synergy</td>
</tr>
<tr>
<td>H358</td>
<td>Bronchioalveolar carcinoma</td>
<td>Null</td>
<td>Mutant</td>
<td>+</td>
<td>Less than synergy</td>
</tr>
<tr>
<td>H322</td>
<td>Adenocarcinoma</td>
<td>Mutant</td>
<td>Mutant</td>
<td>–</td>
<td>Synergy</td>
</tr>
<tr>
<td>H226B</td>
<td>Squamous</td>
<td>Mutant</td>
<td>Wild-type</td>
<td>+</td>
<td>Less than synergy</td>
</tr>
</tbody>
</table>

*The mutation status of p53 in these cell lines was published previously (41, 42).
†† + and – indicate positive and negative expression, respectively, based on the results of western blot analysis.
not statistically significantly greater than the sum of the mean difference of cells treated with each agent alone.

To further investigate the mechanism mediating the antiproliferative effects of Ad-IGFBP3 and SCH66336, we infected H460 cells with Ad-EV, Ad-MEK1 (to generate constitutively active MAPK), or Ad-MyrAkt (to generate constitutively active Akt) and then coinfeected the cells with Ad-EV or Ad-IGFBP3. The induced expression of MEK1 or Akt was confirmed in H460 cells infected with these adenoviral vectors (data not shown), as previously described (30,31). Compared with the growth of control cells (i.e., single adenoviral infection), the growth of cells coinfected with Ad-EV plus Ad-IGFBP3 (65.7% of control, 95% CI = 58.9% to 72.5%; P < .001) and that of cells coinfected with Ad-MEK1 plus Ad-IGFBP3 was statistically significantly reduced (64.4% of control, 95% CI = 56.6% to 73.2%; P < .001) (Fig. 4, B). The growth of cells coinfected with Ad-EV plus Ad-IGFBP3 (31.9% of control, 95% CI = 27.0% to 36.8%; P < .001) and of cells coinfected with Ad-MEK1 plus Ad-IGFBP3 was statistically significantly reduced by the addition of SCH66336 (35.0% of control, 95% CI = 27.9% to 42.1%; P < .001). By contrast, the growth of cells coinfected with Ad-MyrAkt plus Ad-IGFBP3 was not statistically significantly different from growth of cells infected with Ad-MyrAkt, regardless of the presence of SCH66336 (Fig. 4, B).

We tested whether overexpression of constitutively active Akt would rescue H460 cells from apoptosis induced by the expression of Ad-IGFBP3 and treatment with SCH66336. Infection with Ad-IGFBP3 and treatment with SCH66336 induced apoptosis in approximately 80% of uninfected H460 cells or cells infected with Ad-EV but in less than 30% of cells infected with Ad-MyrAkt (Fig. 4, C). In addition, loss of pAkt and of Akt by single-agent treatment or the combination of Ad-IGFBP3 and SCH66336 in H460 cells was completely blocked by infection with Ad-MyrAkt. Because Akt can induce expression of the antiapoptotic protein Bcl-xL (43), we assessed the effect of constitutive Akt activation on Bcl-xL expression. The level of Bcl-xL expression decreased in H460 cells infected with Ad-IGFBP3 and treated with SCH66336 but was similar among cells infected with Ad-MyrAkt, regardless of coinfection with Ad-IGFBP3 and treatment with SCH66336 (Fig. 4, D). Thus, increased apoptosis by infection with Ad-IGFBP3 and treatment with SCH66336 in H460 cells results, in part, from an inhibition of levels and activities of prosurvival Akt and antiapoptotic Bcl-xL proteins.

**Apoptotic Effects of Recombinant IGFBP-3 Protein and SCH66336**

To confirm that the effects we observed with Ad-IGFBP3 were not related to the adenoviral vector itself, we generated a recombinant IGFBP-3 protein fused to a Tat protein, which permits heterologous proteins to enter cells (44). We monitored localization of TAT-BP3 protein into H1299 cells, which have no detectable endogenous IGFBP-3. TAT-BP3 translocation into H1299 cells was evident within 10 minutes, reached a maximum between 10 and 30 minutes, and then rapidly decreased by 3 hours (Fig. 5, A). Nuclear translocation of TAT-BP3 was observed at 10 minutes and reached a maximum at 1 hour. The levels of β-actin and PARP, which were used as cytosolic and nuclear marker proteins, respectively, showed marginal or no detectable change.
We next investigated the growth characteristics of H1299 cells treated with the TAT-BP3 plus SCH66336 or TAT-BP3 plus LY294002 (Fig. 5, A, B). The mean difference in proliferation of cells treated with a combination of TAT-BP3 and SCH66336 (40.1%; 95% CI = 32.4% to 47.9%) was statistically significantly greater than the sum of mean differences of cells treated with TAT-BP3 alone (20.1%; 95% CI = 12.4% to 27.8%) and cells treated with SCH66336 alone (10.4%; 95% CI = 2.6% to 18.4%), \( P = 0.04 \) (Fig. 5, C). Similar antiproliferative effects were observed for cells treated with the combination of TAT-BP3 and LY294002 (10 \( \mu M \), \( P = 0.004 \); 25 \( \mu M \), \( P = 0.005 \)) (Fig. 5, C).

Effects of SCH66336 on IGFBP-3–Induced Antitumor Activities in NSCLC

To determine whether Ad-IGFBP3 and SCH66336 had antitumor activities in vivo, we tested the effect of Ad-IGFBP3 and SCH66336 on NSCLC growth using H1299 xenograft tumors established in athymic nude mice. Mice received a single intratumoral injection of Ad-EV or Ad-IGFBP3, which had no detectable effect on H1299 tumor growth (data not shown). The mice were then treated with SCH66336 or HPCD (a vehicle) for 9 days (Fig. 6, A). Tumor size was measured every day for 16 days. Tumor growth in mice that received Ad-IGFBP3 or SCH66336 alone was not statistically significantly different from tumor growth in the appropriate control groups. By contrast, mice that received the combination of Ad-IGFBP3 and SCH66336 had statistically significantly smaller tumors than mice in the other treatment groups (Fig. 6, A). At day 16, the mean tumor volume in mice that received Ad-IGFBP3 and SCH66336 was approximately 45.2% (95% CI = 36.6% to 53.8%) of that in the mice that received PBS and HPCD (Fig. 6, B). The mean difference in tumor volume for mice who received the combination of Ad-IGFBP3 and SCH66336 (54.8%, 95% CI = 46.2% to 63.4%) was statistically significantly greater than the sum of mean differences in tumor volume for mice who received Ad-IGFBP3 and mice who received SCH66336 (14.3%, 95% CI = 0% to 15.9%) \( P < 0.001 \), indicating that, in H1299 cells, the combination of Ad-IGFBP3 and SCH66336 had increased antitumor activity in vivo.

To determine whether in vivo antitumor activity of the combination of Ad-IGFBP3 and SCH66336 was associated with the induction of apoptosis, we performed TUNEL analysis on H1299 xenograft tumor tissues removed from mice in each
treatment group. The level of TUNEL staining in tissues from mice that received Ad-IGFBP3 and SCH66336 was markedly higher than that in tumor tissues from mice treated with other combinations (Fig. 7, A), demonstrating that treatment with Ad-IGFBP3 and SCH66336 increased apoptosis in H1299 cells in vivo.

Finally, to determine whether the combination of SCH66336 and BP-3 altered Akt and Bcl-xL levels in vivo, we immunohistochemically stained the H1299 xenograft tumor tissues for expression of Akt and Bcl-xL. The levels of Akt and Bcl-xL were lower in tumors from mice that received Ad-IGFBP3 and SCH66336 than in tumors from mice that received other treatment combinations (Fig. 7, B). Thus, these data suggest that Ad-IGFBP3 and SCH66336 can increase the level of apoptosis in H460 and H1299 NSCLC cells, in part, through the inhibition of Akt expression, which results in decreased levels of activated Akt and of Bcl-xL expression.

**DISCUSSION**

In this study, we demonstrated that the combination of SCH66336, a targeted small molecule designed to inhibit Ras farnesylation (25), and IGFBP-3 has growth-inhibitory effects in a subset of NSCLC cell lines. The concentration of SCH66336 (1 μM) is well below the concentration reported to be achievable in vivo (approximately 8 μM) in mice given a single oral dose of 25 mg/kg SCH66336. We found that, in vitro and in vivo, the combination of Ad-IGFBP3 and SCH66336 increased apoptosis, which was associated with decreased Akt and Bcl-xL expression.

Several studies (2–4) have suggested that interventions leading to the disruption of the IGF pathway should induce apoptosis, reverse the transformed phenotype, and have antitumor effects. Among the inhibitors of the IGF pathway, IGFBP-3 might be an effective therapeutic agent for lung cancer because it regulates cell growth through dual mechanisms: IGF-dependent extracellular regulation of antiproliferation and survival pathways and IGF-independent intracellular effects on proliferation and apoptosis (45). We demonstrated that overex-

**Fig. 6.** Effects of combination of adenoviral vector containing insulin-like growth factor binding protein-3 (Ad-IGFBP3) and the farnesyltransferase inhibitor SCH66336 on H1299 non–small-cell lung cancer (NSCLC) xenograft tumor growth. (A) H1299 cells (10^7) were injected into the dorsal flank of athymic nude mice. After tumor volume reached 75 mm^3, mice were randomly assigned to six groups (five mice per group) and given single injection of 2 x 10^9 viral particles of Ad-IGFBP3 or Ad-EV (empty vector) in 100 μL of phosphate-buffered saline (PBS) alone. SCH66336 (40 mg/kg of body weight) was administered orally, twice daily on days 2–10. Proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each bar represents the mean value of six identical wells from a representative single experiment (n = 3). Error bars show upper 95% confidence intervals. * and ** denote P = .04 and P = .004 for TAT-BP3+LY (10 μM); P = .005 for pTAT-BP3+LY (25 μM), respectively. C) Results are expressed as percent cell number relative to the number of PBS-treated control cells.

**Fig. 5.** Effect of combination of recombinant insulin-like growth factor binding protein-3 (TAT-BP3) and the farnesyltransferase inhibitor SCH66336 on the proliferation of H1299 non–small-cell lung cancer (NSCLC) cells. A) H1299 cells were treated with TAT-BP3 (500 ng/mL) for 10, 30, 60, 180, or 360 minutes. The whole-cell lysates (WCE), cytosolic (CE), and nuclear extracts (NE) were subjected to the western blot analysis for the detection of IGFBP-3, HA (hemagglutinin tag for the recombinant protein), β-actin, and poly(ADP-ribose) polymerase (PARP). B) H1299 cells were treated with TAT-BP3 (500 ng/mL) in the absence (--) or presence of SCH66336 (1 μM) or LY294002 (10 or 25 μM) for 3 days. Control cells were treated with the diluent dimethyl sulfoxide (DMSO; 0.1%). Proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each bar represents the mean value of six identical wells from a representative single experiment (n = 3). Error bars show upper 95% confidence intervals. * and ** denote P = .04 and P = .004 for TAT-BP3+LY (10 μM); P = .005 for pTAT-BP3+LY (25 μM), respectively. C) Results are expressed as percent cell number relative to the number of PBS-treated control cells.
pression of IGFBP-3 inhibits NSCLC cell proliferation in vitro and in vivo (9). Recently, recombinant IGFBP-3 protein had single-agent and combinatorial antitumor activity (additive or synergistic) with chemotherapeutic agents (16), indicating therapeutic efficacy.

Despite the potential of IGFBP-3 to be used as a therapeutic agent for lung cancer, several NSCLC cell lines showed mild or no sensitivity to IGFBP-3. In our effort to determine the mechanism underlying nonresponsiveness of these NSCLC cell lines to IGFBP-3, we studied the involvement of Ras-mediated signaling pathway because a recent study (17) implicated a Ras-mediated signaling mechanism in the development of resistance to IGFBP-3. We therefore investigated whether inhibition of the Ras-mediated pathway could increase the antiproliferative effects of IGFBP-3. Indeed, we observed a synergistic antiproliferative effect between Ad-IGFBP3 and SCH66336 in several NSCLC cell lines.

Our in vitro results indicate that the combination of Ad-IGFBP3 and SCH66336 increases apoptosis. The combination of these two agents suppressed expression of antiapoptotic proteins Bcl-2 and Bcl-xL without substantially changing the expression of the proapoptotic protein Bax. The balance between antiapoptotic and proapoptotic proteins has been shown to determine whether a cell survives or undergoes apoptosis (46), suggesting that the increased antiproliferative activities of Ad-IGFBP3 and SCH66336 resulted, in part, from changes in the regulation of the antiapoptotic-to-proapoptotic protein ratio. Because Bcl-xL also functions independently in regulating cell death (47), regulation of the Bcl-xL protein expression could, in part, account for the increased apoptosis in NSCLC cells treated with Ad-IGFBP3 and SCH66336.

The level of IGFBP-3 expression (34) did not affect the responsiveness of NSCLC cells to SCH66336 (Fig. 2). IGFBP-3 activity is altered by posttranslational modifications, including proteolysis; proteases such as kallikrein-like serine proteases, cathepsins, and matrix metalloproteinases (MMPs) cleave IGFBP-3 into small fragments that have reduced affinity for IGFs (48–50). Increased MMP activity has been consistently detected in malignant tissues, including lung tumors (51), suggesting that activated MMPs may cleave and inactivate IGFBP-3 in lung cancer. IGFBP-3 has also been detected as a phosphoprotein that influences ligand binding (52,53). IGFBP-3 has potential phosphorylation sites for casein kinase II, cyclic adenosine monophosphate–dependent protein kinase, calcium/phospholipid–dependent protein kinase, and MAPK, all of which can be activated by the Ras-mediated signaling pathway (52). Therefore, it is reasonable to speculate that, in some NSCLC cell lines, endogenously expressed IGFBP-3 could be inactivated by these modifications and thus SCH66336 would have shown no stimulating effects on IGFBP-3-mediated apoptosis. Although overexpressed IGFBP-3 induced by the adenoviral vector would presumably be modified by the same proteases and kinases, it is possible that IGFBP-3 levels in such cells reached cytotoxic levels and induced apoptosis.

We investigated the mechanism underlying the increased apoptosis mediated by Ad-IGFBP3 and SCH66336. Although SCH66336 targets the Ras signaling pathway and most FTIs block the in vitro growth of transformed cells with wild-type Ras (26), the response of NSCLC cell lines to Ad-IGFBP3 and SCH66336 was not associated with Ras gene mutations (Table 1). Thus, inhibition of Ras activation by SCH66336 may not adequately account for the increased apoptotic effects of Ad-IGFBP3 and SCH66336 in NSCLC cell lines. In support of this notion, recent studies (26,27) have demonstrated that some FTIs have antitumor activities that are independent of Ras. Jiang et al. (54) demonstrated that FTIs block the PI3K/Akt-mediated
growth factor—and the adhesion-dependent survival pathway and induce apoptosis in human cancer cells that overexpress Akt. In that study, overexpression of Akt2 but not of oncogenic H-ras sensitized NIH/3T3 cells to the TFI. Therefore, we studied the involvement of downstream mediators of Ras-mediated signaling pathways to understand the mechanism of action of SCH66336. We found that, in H460 cells infected with Ad-IGFBP3 and treated with SCH66336, Akt expression was inhibited as a result of decreased protein stability and that inhibition of Akt expression increased apoptosis. This conclusion is supported by several lines of evidence. First, overexpression of constitutively active Akt but not constitutively active MEK1 blocked H460 cells from undergoing apoptosis induced by Ad-IGFBP3 and SCH66336. Second, the combination of Ad-IGFBP3 and SCH66336 blocked expression of the antiapoptotic protein Bcl-xL, which is regulated by Akt (43), except in cells that overexpressed constitutively active Akt. Third, the inhibitory effects on NSCLC cell growth, increased apoptosis, and decreased expression of Akt and Bcl-xL by Ad-IGFBP3 and SCH66336 were also observed in vivo in established NSCLC xenograft tumors established, suggesting the therapeutic efficacy of this combination.

In conclusion, our results demonstrate that the apoptotic activities of IGFBP-3 are increased by SCH66336 in NSCLC cells both in vitro and in vivo. Although inhibition of Ras activation by SCH66336 and blockade of IGF signaling by IGFBP-3 could contribute to cell death, decreased Akt stability seems to be one mechanism underlying the increased apoptotic activities of the IGFBP-3 and SCH66336 combination. LY294002, a PI3K/Akt inhibitor that blocks ATP binding to the p110α PI3K catalytic domain (55), was also effective in increasing the antiproliferative activities of Ad-IGFBP3 and of TAT-BP3. These findings suggest that the PI3K/Akt pathway might be involved in regulating antiproliferative activities of intracellular IGFBP-3, and they implicate IGFBP-3 in a mechanism regulating Akt stability. Akt is constitutively active in most NSCLC cells (9,56), probably by multiple mechanisms (18–24), and could interfere with the functions of intracellular IGFBP-3. Therefore, suppression of PI3K/Akt activity might be required in designing IGFBP-3–based therapeutic strategies for NSCLC. In support of this idea, recombinant IGFBP-3 protein (TAT-BP3) has enhanced antiproliferative effects on NSCLC cells, when combined with SCH66336 or LY294002.

The role of the IGFBP-3 and SCH66336 combination as an inhibitor of Akt expression has clinical implications, especially for NSCLC, in which constitutive Akt activation occurs frequently (56). Ongoing work is directed toward delineating the optimal clinical protocol in which to translate the activity of IGFBP-3 observed in vitro and in vivo. In addition, because tumor growth was not completely suppressed by simultaneous treatment of Ad-IGFBP3 and SCH66336, additional studies on optimal dosing schedule of the combination treatment and the sequence of administration of two agents, which affect the degree of synergism and thus the therapeutic efficacy (36), are warranted.

REFERENCES


NOTES

Supported by National Institutes of Health Grants ROI1 CA100816-01A1 (to H.-Y. Lee), American Cancer Society, RSG-04-082-01-TBE 01 (to H.-Y. Lee), M. D. Anderson Cancer Center institutional grants Goodwin Funds for Targeted Molecular Diagnosis and Therapeutics (to H.-Y. Lee), DAMD17-01-1-0689 from the Department of Defense (to W. K. Hong), W81XWH-04-1-0142-01-VITAL from the Department of Defense (to W. K. Hong). W. K. Hong is an American Cancer Society clinical research professor.

Manuscript received January 20, 2004; revised July 26, 2004; accepted August 20, 2004.