Published in final edited form as:

Cancer Res. 2008 February 15; 68(4): 981–988. doi:10.1158/0008-5472.CAN-06-0249.

Growth Suppression of Lung Cancer Cells by Targeting Cyclic AMP Response Element-Binding Protein

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Abstract

Genes regulated by cyclic AMP response element-binding protein (CREB) have been reported to suppress apoptosis, induce cell proliferation, and mediate inflammation and tumor metastasis. However, it is not clear whether CREB is critically involved in the carcinogenesis of lung cancer. We found that non-small cell lung cancer (NSCLC) cell lines exhibited elevated constitutive activity in CREB; in its immediate upstream kinases, ribosomal s6 kinase and extracellular signal kinase; and in the CREB-regulated cell survival proteins, Bcl-2 and Bcl-xL. We hypothesized that constitutively active CREB is important to lung cancer cell growth and survival and therefore could be a potential therapeutic target for NSCLC. Ectopic expression of dominant-repressor CREB and transfection with small interfering RNA against CREB suppressed the growth and survival of NSCLC cells and induced apoptotic cell death. Furthermore, treating H1734 NSCLC cells with an inhibitor of the CREB signaling pathway, Ro-31-8220, inhibited CREB activation by blocking the activity of extracellular signal kinase and ribosomal s6 kinase, arrested the cell cycle at the G2/M phase, and subsequently induced apoptosis with the suppression of Bcl-2 and Bcl-xL expression. Ro-31-8220 suppressed both the anchorage-dependent and the independent growth of NSCLC cells, but its cytotoxic effect was much less prominent in normal bronchial epithelial cells. Our results indicate that active CREB plays an important role in NSCLC cell growth and survival. Thus, agents that suppress CREB activation could have potential therapeutic value for NSCLC treatment.

Keywords

Lung Cancer; CREB; Growth; Apoptosis; Survival

Introduction

Despite the availability of assorted standard treatments, such as surgery, radiotherapy, chemotherapy, or combined regimens, non-small cell lung cancer (NSCLC) remains an aggressive lung cancer associated with a poor patient survival. Hence, there is an imminent need for better therapies for NSCLC. Recently, the advent of targeted therapy offers new hope to various subsets of cancers by targeting specifically the signal transduction involving carcinogenesis and tumor growth (1-3). Thus, identifying new molecular targets for treatment and/or prevention of NSCLC is warranted and urgently needed to improve the control of this deadly form of lung cancer.

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Research during the past few years has demonstrated that transcription factor cyclic AMP response element-binding protein (CREB) regulates the expression of a repertoire of genes related to cell survival (4,5), inflammation (6), and proliferation (7), such as Bcl-2, Bcl-xL, cyclo-oxygenase-2, and tumor necrosis factor alpha (TNF- α). CREB has also been shown to involve the carcinogenesis of several types of cancer. For example, inhibition of CREB-dependent transcriptional activity using CRE-decoy oligonucleotide caused antiproliferative effects on cancer cells by inhibiting aromatase expression in breast adipose tissue (8). Furthermore, studies using ectopic expression of dominant-repressor CREB (KCREB) demonstrated that in androgen-dependent prostate cancer cells, dihydrotestosterone stimulation caused an anti-apoptotic effect through the phosphorylation of CREB (9). CREB was found to be overexpressed in bone marrow cells from patients with acute lymphoid or myeloid leukemia, suggesting that CREB is involved in leukemogenesis and that its overexpression can be a biomarker for leukemia (7,10-12). Finally, in human melanoma cells, CREB has been reported to be a mediator of tumor growth and metastasis, and the expression of KCREB sensitizes melanoma cells to apoptosis and inhibits their growth and metastasis (13-17).

Although it is not known whether CREB is directly involved in lung cancer, several lines of evidence have supported this possibility. For example, a murine spontaneous lung tumors model induced by transgenic over expression of insulin-like growth factor (IGF)-II displayed high levels of phosphorylated CREB in such tumor tissue, and IGF-II-induced CREB phosphorylation played an important role in survival of lung cancer cell line (18,19). NSCLC cells often overexpress Bcl-xL and Bcl-2 (20), which are regulated by CREB, and their suppression can result in apoptosis. Moreover, a recent study exploring the role of neurotransmitter receptor on tumorigenesis have found that blockade of the receptor suppressed the growth of lung carcinoma cells by reducing the phosphorylation of CREB and the expression of CREB-regulated genes, such as *c-fos*, *c-jun*, *bcl-2* and *cyclin D1* (21). Thereof, we aim to determine whether constitutively active CREB (phosphorylated CREB; pCREB) is a potential target for treating NSCLC and whether its inhibition blocks cell proliferation and induces apoptosis in NSCLC cells.

CREB is an effector for a variety of receptors, such as receptors for growth factors, hormones, retinoids, cytokines, and prostaglandins. It can be activated via multiple pathways by various upstream kinases, including protein kinase A (PKA) (22,23), protein kinase C (PKC) (24), MAPK activated protein-2 (25), Akt (26), and CaM KII and IV (27,28). Ro-31-8220 is a well-known inhibitor of PKC, and it was also found to inhibit p90 ribosomal S6 kinase (p90Rsk) and mitogen and stress activated kinase (Msk) (29,30). As PKC, Rsk and Msk are all important upstream activators of CREB, we surmise that this compound would in effect inhibit CREB. We use this compound in addition to other genetic tools to study the effect of CREB on tumor cell growth and to evaluate the potential of targeting CREB signaling as a strategy for cancer therapy.

Materials and Methods

Cell Culture

We obtained four human NSCLC cell lines, H1734 (lung adenocarcinoma), H226 (lung squamous cell carcinoma), A549 (alveolar lung epithelium cell, poorly differentiated), and H292 (pulmonary mucoepidermoid adenocarcinoma), from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI containing 10% FBS. Normal human tracheobronchial epithelial (NHTBE) cells (Clonetics, San Diego, CA), which were used as the control, were cultured by a three-dimensional organotypic air-liquid interface method as described previously (31-35). Ro-31-8220 was purchased from Calbiochem (La Jolla, CA) and prepared in DMSO.

Western Blot Analysis

The whole cell lysate were prepared by lysing the cells in SDS lysis buffer (250 mM Tris-Cl, pH 6.5, 2% SDS, 4% β -mercaptoethanol, 0.02% bromophenol blue, 10% glycerol) containing protease and phosphatase inhibitors. Standard SDS-PAGE and western blotting procedures were used to analyze the cell lysate. Blots were probed with anti-CREB and anti-phospho-CREB (Ser-133) (Upstate Biotechnology, Waltham, MA); anti-Bcl-2, anti-Bcl-xL, anti-phospho-Erk1/2, and anti-Erk1/2 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Rsk and anti-phospho-Rsk (Cell Signaling Technology, Cambridge, MA). To detect the cleavage products of apoptosis, blots were probed with anti-poly (ADP-ribose) polymerase (PARP), anti-caspase-9, and anti-caspase-3 antibodies (New England Biolabs, Beverly, MA). Anti-actin antibodies were from Sigma - Aldrich (St. Louis, MO) and the caspase-3 inhibitor Ac-DEVD-CHO was from Promega (Madison, WI).

Electrophoretic Mobility Shift Assay (EMSA)

Isolation of nuclear extracts, and preparation of CRE oligonucleotide probes, were conducted as previously described (36). For super-shift analysis, the nuclear extracts from H1734 cells were incubated with radio labeled oligonucleotide probes in the presence of antibodies against CREB or pCREB for 30 min at 37°C. Unlabeled (cold) probe and a CRE mutant oligonucleotide 5'-AGAGATTGCCTGTGGTCAGAGAGCTAG -3' (bold face: mutated bases; Santa Cruz Biotechnology, CA) (100-fold) was used to check the specificity of the probes. The radioactive bands on the dried gels were visualized and quantitated with Phospho-Imager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software (Amersham Biosciences, Piscataway, NJ).

Immunocytochemistry for CREB Localization

H1734 cells (1×10^5 cells/ml) were plated on a glass chamber slide (Falcon; BD Biosciences, San Jose, CA) and treated the next day for 4h with Ro-31-8220. The chambers were removed, and cells were fixed with cold acetone. After a brief washing with PBS, the slides were blocked with 5% normal goat serum in PBS for 1h and then incubated for 4h with rabbit anti-pCREB antibody (1:100 dilution). The slides were washed, incubated with goat anti-rabbit IgG-Alexa 594 (Molecular Probes, Eugene, OR) (1:100 dilution) for 1h, and counter-stained for nuclei with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; 50 ng/ml) (Molecular Probes, Eugene, OR) for 15 min. The slides were mounted, and confocal images were captured with an Olympus FV500 confocal microscope (CenterValley, PA) by using a Pl an Apo 60 $\times$ oil objective.

Transfection and Cytotoxicity Assay

H1734 cells (10,000/0.5ml) were plated in triplicate in 24-well plates and transfected with plasmid DNA (50 ng/well) of wild type CREB (CREBwt), KCREB, and CREB133 using lipofectamine 2000 (Invitrogen, Carlsbad, CA). For RNA interference, H1734 cells were transfected with a final concentration of 100 nM siRNA SMARTpool sequences targeting human CREB or non-specific control pool (Dharmacon RNA Technologies, Lafayette, CO) using siIMPORTER siRNA transfection reagent (Upstate Biotechnology, Waltham, MA). The transfection efficiencies of the control plasmid were consistently above 80%. After 48 h of expression of CREBwt, KCREB, CREB133, or siCREB, the cytotoxic effect was determined with the MTT method as described by the manufacturer (Sigma, St. Louis, MO). The cytotoxic activity of Ro-31-8220 was also determined on the all four NSCLC cells and NHTBE cells. Briefly, the cells (2,000/well) were incubated in triplicate in 96-well plates in the presence or absence of indicated agent in a final volume of 0.2 ml for different days at 37°C. Afterwards, we performed the MTT assay.

Flow Cytometric Analysis

H1734 cells were synchronized by serum starvation overnight before exposure to Ro-31-8220 in the presence of 5% serum. NHTBE cells were grown in air-liquid-interface and treated with Ro-31-8220 in their normal medium. After different lengths of time, cells were washed, trypsinized, and fixed in 70% ethanol for 1 h at -20°C . Finally, cells were washed with PBS and stained with propidium iodide (25 $\mu\text{g}/\text{ml}$) in the presence of 1 $\mu\text{g}/\text{ml}$ RNAase for 30 min at room temperature. The distribution of cells in the cell cycle was then analyzed with a FacsCalibur flow cytometer (Becton Dickinson, Bedford, MA).

Soft Agar Colony Formation Assay

Single-cell suspensions of the H1734 and H226 cells were treated with or without different concentrations of Ro-31-8220 and then mixed with agarose to a final concentration of 0.35%. Aliquots of 1.5 ml containing 10^4 cells and 10% FBS were plated in triplicate in six-well plates over a base layer of 0.7% agarose and allowed to gel. The number of colonies greater than 60 μm was counted after 28 days of incubation.

Apoptosis Assay

H1734 cells (200,000) were plated in chamber slides; the next day, transfected with plasmids encoding CREBwt, KCREB, or CREB133 (100 ng DNA/chamber), or with 100 nM siCREB. The terminal transferase dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer's instructions (Promega). Briefly, 48 h after transfection the cells were fixed in 4% formaldehyde and permeabilized with 0.5% Triton-X-100. The cells were then incubated with terminal deoxynucleotidyl transferase and fluorescein-dUTP, and co-stained with DAPI and visualized with an Axioskop 40 fluorescence microscope (Carl Zeiss, Thornwood, NY). Three random fields were counted for each sample. The images were captured at a magnification of 400 $\times$ and stored using Axiovision software (Carl Zeiss).

Statistical analysis

Statistical analysis was performed with Prism program (GraphPad Software, San Diego, CA). For multiple groups comparison, one-way analysis of variance (ANOVA) was used, followed by Dunnett's test for comparing experiment groups against a single control. For single comparison between two groups, Student's *t*-test was used.

Results

CREB Signaling Is Constitutively Overactivated in NSCLC Cells as Compared with NHTBE Cells

To compare the constitutive CREB signaling between tumor and normal cells, we measured the expression level of CREB and its activation status among different cell lines under unstimulated normal growth condition. Western blot analysis showed that both CREB and p-CREB levels were substantially higher in all four NSCLC cell lines as compared with NHTBE cells (Fig. 1A).

To determine whether such elevation in CREB phosphorylation was indeed translated into the CRE-dependent transcriptional activity, we performed an EMSA with radio-labeled oligonucleotide probes containing consensus CRE motif (5'-TGACGTCA-3'). We found that nuclear extracts from all four NSCLC cell lines contained higher amount of CRE binding proteins than that from NHTBE cells as indicated by the intensity of the single retarded band (Fig. 1B). The specificity of the probes was verified with 100-fold excess of unlabeled (cold) consensus CRE and mutant CRE oligonucleotides (lanes 3 and 4, Fig. 1C) using the nuclear

extract from H1734 cells. Further retention of the radio-labeled band (super shift) by anti-CREB and anti-pCREB but not control IgG (*lanes 2, 5 and 6, Fig. 1C*) confirmed that the previous single band resulted from the interaction between CRE oligonucleotide and activated CREB.

CREB Activity Is Inhibited by Ro-31-8220 in H1734 Cells

Ro-31-8220 substantially suppressed CREB activation in a time- and dose- dependent manner in the H1734 cells (*Fig. 2A*). Moreover, the CRE-binding capacity of nuclear extract from H1734 cells was also suppressed by Ro-31-8220 in a time- and dose-dependent manner (*Fig. 2B*). H1734 cells treated with or without Ro-31-8220 for 4 h were fixed, and immunocytochemically stained with anti-pCREB antibody to analyze the cellular distribution of pCREB. Confocal images of the staining showed that Ro-31-8220 induced the nuclear disappearance of pCREB in the H1734 cells (*Fig. 2C*).

Upstream and Downstream Components of CREB Signaling Are Constitutively Overactivated in NSCLC Cells and Their Activation Can Be Inhibited by Ro-31-8220

Rsk is the major kinase that directly phosphorylates CREB. Our western blot analysis showed that all four NSCLC cell lines exhibited higher basal level of phospho-Rsk relative to the NHTBE cells (*Fig. 3A, upper*). Incubation of the H1734 cells with Ro-31-8220 (20 μ M) suppressed the phospho-Rsk level within 2 h (*Fig. 3B, upper*); such suppression became noticeable at a concentration of 5 μ M for 4 h treatment of Ro-31-8220 (*Fig. 3C, upper*). Moreover, Erk1/2, an important upstream kinase for Rsk activation is also constitutively over activated in all four NSCLC cell lines as compared with the NHTBE cells (*Fig. 3A, middle*). The phospho-Erk1/2 level was decreased by Ro-31-8220 treatment within 30 min in H1734 cells (*Fig. 3B, middle*); this decrease was detectable since 5 μ M of Ro-31-8220 treatment (*Fig. 3C, middle*).

The cell survival signaling regulated by CREB transcription factor was also examined. Western blot results showed that all four NSCLC cell lines constitutively overexpressed Bcl-xL and Bcl-2 as compared with the NHTBE cells (*Fig. 3A, lower*). Such overexpression can be suppressed by Ro-31-8220 treatment in a time- and dose-dependent fashion (*Fig. 3B and C, lower*).

Ro-31-8220 Suppresses Cell Growth and Tumorigenicity in NSCLC Cells

The effect of Ro-31-8220 on cell proliferation was then evaluated with MTT assay. The results showed that Ro-31-8220 suppressed cell proliferation in all four NSCLC cell lines in a dose-dependent manner (*Fig. 4A*). Such suppression on cell proliferation, however, was significantly less in NHTBE cells (*Fig. 4B*). Taken together, these results showed that Ro-31-8220 had a more pronounced cytotoxic effect on the NSCLC cells than on the NHTBE cells. Other than the effect on anchorage-dependent cell growth, we also examined the effect of Ro-31-8220 on the anchorage-independent growth of NSCLC cells, which is an indicator of tumorigenicity, using a soft agar colony formation assay. Our results showed that Ro-31-8220 significantly inhibits the colony formation in both H1734 and H226 cells at 2 μ M concentration (*Fig 4C*). The effect of Ro-31-8220 on NHTBE cells was not included for comparison here as the cells cannot form colony in soft agar (data not shown).

Ro-31-8220 Induces G2/M Cell Cycle Arrest and Apoptosis in NSCLC Cells

To delineate the mechanism by which Ro-31-8220 inhibit cell growth, we examined the effect of Ro-31-8220 on the cell cycle of H1734 and NHTBE cells. After 12 h incubation, the percentage of cells in the G2 phase increased from 13% in the controls to 29% in the Ro-31-8220-treated H1734 cells and after 24 h, the percentage of cells in G2 phase

increased from 17% in the controls to 23% in Ro-31-8220 treated cells (Fig. 5A). These results indicated that Ro-31-8220 induced G2/M arrest in H1734 cells. However, the percentage of cells in the G2 phase was not increased by Ro-31-8220 treatment in NHTBE cells (12 h: control, 17%, treated, 13%; 24h: control, 18%, treated, 15%). Noticeably, after 24 h treatment, the percentage of H1734 cells in sub-G1 phase increased from 1% to 16%, whereas the percentage of NHTBE cells in sub-G1 phase remained less than 2%.

As cell cycle arrest is a precursor of apoptosis, we examined the signature cleavage of apoptosis-related proteins in H1734 cells. In Ro-31-8220 treated cells, western blot results showed the cleavage of a 118-kDa PARP protein into an 87-kDa fragment, a hallmark of cells undergoing apoptosis, whereas in untreated cells the protein was not cleaved (Fig. 5B). The Ro-31-8220 induced apoptosis was further evidenced by a time-dependent activation of caspase-9 (Fig. 5B) as indicated by the cleavage of a 47-kDa band to a 37-kDa band. Similarly, the blots showed the activation of caspase-3 by Ro-31-8220 (Fig. 5C, *lane 3*) through the cleavage of a 37-kDa band to a 16-kDa band. These findings demonstrate that Ro-31-8220 induced apoptosis in the H1734 cells. To determine whether caspase activation is required for Ro-31-8220 induced suppression of cell growth, we used a caspase-3 inhibitor, Ac-DEVD-CHO, to block caspase activation. The effectiveness of Ac-DEVD-CHO in blocking the Ro-31-8220 induced caspase-3 cleavages was confirmed on Fig. 5C. H1734 cells were then treated with Ro-31-8220 in the presence of different concentrations of Ac-DEVD-CHO, and cell viability was assessed with MTT assay. The caspase-3 inhibitor effectively protected the cells from Ro-31-8220 induced cytotoxicity at a concentration as low as 0.01 μ M. (Fig. 5D). These results suggest that caspase-3 activation is essential for Ro-31-8220 induced cytotoxicity.

Suppression of CREB Expression and Activation Inhibits Cell Proliferation, and Induces Apoptosis in NSCLC Cells

We have demonstrated that Ro-31-8220 inhibited constitutively active CREB, suppressed cell proliferation, and induced apoptosis. To test whether the inhibition of constitutively active CREB is accountable for the anti-tumorigenesis effect of Ro-31-8220, we suppressed the constitutive CREB activity in H1734 cells by knockdown of CREB expression with RNA interference, or by repressing the CREB transcriptional activity with the transfection of KCREB and CREB133, and observed the effect on cell proliferation. Our results showed that suppression of constitutively active CREB by all three methods inhibited cell growth (Fig. 6A and B). In contrast, transfection with CREB wild type or non-target siRNA did not affect cell growth. The efficient siRNA knockdown of CREB and the ectopic expression of KCREB and CREB133 were confirmed by western blots analysis (Fig. 6C, *upper*). Next, we examined whether such genetic quenching of constitutive CREB activity induced apoptosis. We found that suppression of CREB activity induced cleavage of both PARP and caspase 3 as did in Ro-31-8220 treatment (Fig. 6C). To confirm the effect of suppression of CREB activity on apoptosis, we performed a TUNEL assay. As expected, suppression of constitutive CREB activity caused a significant increase in the percentage of TUNEL positive cells in H1734 cells—44% for CREB-siRNA, 35% for KCREB, and 39% for CREB133, respectively—compared with controls (6% for CREBwt and 8% for non specific siRNA) (Fig. 6D). Meanwhile, treatment with 5 μ M Ro-31-8220 for 48 h induced apoptosis in 52% of the cells. These data suggest that the depletion of CREB in the H1734 cells sensitized the cells to apoptosis.

Discussion

CREB is an important transcription factor that involves numerous physiological processes, including differentiation of bronchial epithelial cells (36,37). Aside from the physiological importance, the role of CREB in tumorigenesis has also been indicated lately (see

Introduction). In the present study, we found that CREB is overexpressed and constitutively active in several human NSCLC cell lines. Moreover, we found that direct and/or indirect suppression of constitutively active CREB inhibits cell proliferation and induces apoptosis in NSCLC cells.

Several different upstream pathways have been known to lead to CREB activation. Among them, PKC pathway has been shown to induce CREB activation via a mitogen-activated protein kinases (MAPK) cascade (36,37). Based on the inhibitory effect of Ro-31-8220 on several components along the PKC-CREB pathway, we used this compound to suppress CREB activation, and found that Ro-31-8220 effectively inhibited the CREB activity (Fig. 2) and the growth of all four NSCLC cell lines we tested (Fig. 4A). Furthermore, the upstream and downstream signaling of CREB, including the phosphorylation of Erk and Rsk, and the expression of Bcl-2 and Bcl-xL, are also effectively suppressed (Fig. 3B and C). These results not only demonstrated the importance of CREB but also indicated that CREB may be constitutively activated by the PKC branch of its upstream pathway in the NSCLC cells. This assumption is further supported by the observation that the activity of two signaling components in the PKC-CREB pathway, ERK1/2 and RSK, are also markedly higher in the four tumor cell lines as compared with normal cells (Fig. 3A). Our results agree with the report that phospho-Rsk and phospho-Erk1/2 are involved in the CREB activation (38,39) and our own studies that PKC is involved in CREB signaling pathway (36).

As mentioned earlier, CREB plays a pivotal role in cellular signaling, and several upstream signaling pathways converge on CREB (40). Therefore, regardless the upstream pathways, CREB appears to be the most effective target for inhibiting the CREB-associated tumor cell growth. In addition, the expression of *CREB* gene can be up-regulated by the activation of CREB *per se* (41). Such an effect might account for the higher level of CREB observed in the NSCLC cells (Fig. 1A), and would also make the CREB level more effectually modulated. Other than the strategic advantage, the most compelling rationale for CREB targeting is the mechanism by which CREB supports tumor cell growth. We found that the expression of the CREB-regulated anti-apoptotic proteins, Bcl-2 and Bcl-xL, is significantly higher in NSCLC cells as compared with NHTBE cells (Fig. 3A), and such overexpression can be effectively suppressed by blocking the CREB signaling (Fig. 3B and C). Our findings agree with other reports that Bcl-2 and Bcl-xL are regulated by CREB(42). Moreover, the inhibition of CREB activity either by knockdown of CREB expression level with siCREB or expression of dominant negative form of CREB mutants (KCREB and CREB133) induced apoptosis in NSCLC cells. We verified this apoptotic effect with various assays, including flow cytometry analysis, the signature cleavage of PARP and caspases, and TUNEL assay (Fig. 5 and 6). The rescue of such cell death by a caspase inhibitor, Ac-DEVD-CHO, affirmed that the major cause of cell death is apoptosis (Fig. 5C and D). Other studies have shown that the expression of KCREB decreases the tumorigenicity and tumor metastatic potential in nude mice (15,16,43), reduces the resistance of human melanoma cells to radiation (13), and reduces adipogenesis (44). In addition, the ability of forming colony in soft agar, a measure of aggressive cell growth, was clearly inhibited in NSCLC cells at a lower concentration of Ro-31-8220 (Fig. 4C). Conversely, NHTBE cells are not as susceptible as NSCLC cells to the cell death induced by the inhibition of CREB (Fig. 4B).

Concomitantly, we have observed in archived lung tumor specimens that the expression levels of CREB and p-CREB are distinctively higher in tumor tissues relative to the adjacent normal tissues, and that such overexpression is inversely correlated with the survival duration of a subset of NSCLC patients (data submitted in a separate manuscript). Our results indicate that NSCLS cells are highly dependent on the elevated CREB activity for their survival and cell growth, and inhibition of CREB effectively suppress the growth of

NSCLC cells. Taken together, over activation of CREB confers a CREB-dependent survival and cell growth on NSCLC cells. Such dependence on the excessive CREB activity furnishes an extended therapeutic window for targeting CREB as a strategy for treating NSCLC tumors.

Acknowledgments

Grant support: National Heart, Lung, and Blood Institute grant R01-HL-077556 (to J.S. Koo), Department of Defense VITAL grant W81XW-04-1-0142 (to J.S. Koo), Head and Neck Specialized Program of Research Excellence (P50 CA097007), Developmental Research Program Grant (to J.S. Koo), and National Cancer Institute Cancer Center Support Grant CA-16672 (to The University of Texas M. D. Anderson Cancer Center).

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Figure 1.

CREB was overexpressed and constitutively activated in NSCLC cells. *A*, Whole cell lysates were made from NHTBE, H1734, H226, H292, A549 cells and tested for constitutive levels of pCREB and total CREB by western blot analysis. The same blot was stripped and re-probed with an anti- β -actin antibody to show equal loading of samples (*lower panel*). *B*, Nuclear extracts from NHTBE, H1734, A549, H226, and H292 cells were prepared as described in Methods and then assayed for CREB activation by EMSA with a radio-labeled CRE consensus oligonucleotide probe. *C*, To verify the specificity of EMSA analysis, nuclear extracts from H1734 cells were incubated with CRE probe in the presence of anti-pCREB or anti-CREB antibodies or 100 fold unlabeled (cold) CRE oligonucleotide or mutant CRE oligonucleotide or non specific Immunoglobulin G (IgG), and then the band pattern change was examine in EMSA. *: Shifted band. The representative gel images from three independent experiments are shown.

Figure 2.

Ro-31-8220 inhibited constitutively active CREB in NSCLC cells. **A.** H1734 cells were treated with 20 μ M Ro-31-8220 for the designated times, or with various concentrations of Ro-31-8220 for 4 h. The whole cell extracts were analyzed with western blot using anti p-CREB. In each case, the same blot was stripped and re-probed with the anti-CREB antibody (lower panel in each figure). **B.** The nuclear extract from H1734 cells received the above treatments were subjected to EMSA for CRE-binding ability. **C.** Ro-31-8220 induced the disappearance of nuclear pCREB in NSCLC cells. H1734 cells were incubated with or without 20 μ M Ro-31-8220 for 4 h and then analyzed for pCREB with immunocytochemistry. The green stain indicates the localization of pCREB, and the blue

stain indicates the localization of the nucleus. The images shown are representatives of three independent experiments.

Figure 3.

Upstream and downstream components of CREB signaling are constitutively over-activated in NSCLC cells and their activation is suppressed by Ro-31-8220. **A.** Whole cell lysates from NSCLC cell lines (H1734, H226, H292, and A549) and NHTBE cells were analyzed with western blot for the levels of phospho-Rsk and phospho-Erk1/2, and the expression of cell survival gene, Bcl-2 and Bcl-xL. **B.** and **C.** H1734 cells were treated with 20 μ M Ro-31-8220 for the indicated times or with various concentrations of Ro-31-8220 for 4 h, and the whole cell lysate was assessed for the levels of signaling components mentioned above. Equal sample loading is indicated by the band intensity of the inactive form of each protein or β -actin (*lower panel* in each figure).

Figure 4.

Ro-31-8220 suppresses cell growth and colony formation in NSCLC cells. **A**, Cells (H1734, H226, A549 and H292) were plated in 96-well plates and incubated in triplicate with the indicated dose of Ro-31-8220 for different days. Cell proliferation was assessed using MTT assays. The results are shown as the means ($\pm$ standard error) of three independent experiments. **B**, The effect of Ro-31-8220 (10 μ M) on cell viability was compared between NHTBE and NSCLC (H1734 and H226) cells using MTT assay as mentioned above. The results are shown as the means ($\pm$ standard error) of three independent experiments. **C**, H1734 and H226 cells were seeded in 0.35% agar and treated with different concentrations of Ro-31-8220. Colonies formed after 28-day incubation at 37°C in 5% CO₂ were counted under light microscopy. Colonies greater than 60 μ m were scored. Result is mean ($\pm$ standard error) of three independent experiments. **: $p < 0.01$.

Figure 5.

Ro-31-8220 leads to G2/M cell cycle arrest followed by apoptosis in NSCLC cells. **A**, Serum-starvation synchronized H1734 cells and NHTBE cells were incubated in the absence or presence of 10 μ M Ro-31-8220 for the indicated times. Then, cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. Data (means $\pm$ standard error) shown are percentages of cells in each phase of cell cycle from three experiments. *: $p < 0.05$; **: $p < 0.01$ (one-tailed, t -test). **B**, H1734 cells were incubated with or without 20 μ M Ro-31-8220 for the indicated times in medium containing 5% of serum. Whole cell lysates were subjected to western blot analysis using anti-PARP, anti-caspase-9 antibodies. **C**, Suppression of Ro-31-8220 induced caspase-3 cleavage by caspase-3 inhibitor. H1734 cells were pre-incubated with and without caspase inhibitor 5 μ M Ac-DEVD-CHO for 2 h and then treated with 20 μ M Ro-31-8220 for 24 h. Then, cell extracts were prepared and analyzed for caspase-3 cleavage by Western blot analysis using an anti-caspase-3 antibody. DMSO was used as a vehicle control. **D**, The caspase-3 inhibitor protects cells from Ro-31-8220 induced cytotoxicity. H1734 cells were incubated with different concentrations of caspase inhibitor Ac-DEVD-CHO as indicated for 2 h and then treated with 20 μ M Ro-31-8220. After 24 h, cell viability was determined by the MTT. The results are shown as the means ($\pm$ standard error) percentage viability from triplicate cultures of three independent experiments. **: $p < 0.01$.

Figure 6.

Suppression of CREB inhibits cell proliferation and induces apoptosis. **A**, H1734 cells were transfected with CREBwt, KCREB, and CREB133 plasmid, and then assayed for cell proliferation by the MTT assays. The results are shown as the means ($\pm$ standard error) of three independent experiments. **B**, H1734 cells were transfected with siCREB and non-specific control pool siRNA (NS-siRNA), then assayed for cell proliferation by the MTT assays. The results are shown as the means ($\pm$ standard error) of three independent experiments. **C**, H1734 cells were transiently transfected with siCREB, siRNA-NS, CREBwt, KCREB, and CREB133 plasmid. Three days after transfection, equal amounts of whole-cell lysates were prepared and subjected to western blot analysis using the indicated antibodies. **D**, TUNEL analysis was performed on H1734 cells 48 h after transfection with the indicated plasmids or treatment with Ro-31-8220 (5 μ M). Nuclei appear blue (DAPI stain), TUNEL-positive nuclei stain green. Magnification: 200x. The results are shown as the means of three independent experiments.