Tumorigenesis and Neoplastic Progression

Activated cAMP Response Element Binding Protein Is Overexpressed in Human Mesotheliomas and Inhibits Apoptosis

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Little is known about the cellular mechanisms contributing to the development and chemoresistance of malignant mesothelioma (MM), an aggressive asbestos-associated tumor. A human mesothelial cell line (IP9/TERT-1) and isolated human pleural mesothelial cells showed rapid and protracted asbestos-induced cAMP response element binding protein (CREB1) phosphorylation, which was inhibited in IP9/TERT-1 cells by small molecule inhibitors of epidermal growth factor receptor phosphorylation and protein kinase A. Asbestos increased expression of several CREB target genes (*c-FOS*, *EGR-1*, *MKP1*, *BCL2*, and *MMP13*) and apoptosis, which was enhanced using small interfering CREB. Human MM tissue arrays showed elevated endogenous levels of phosphorylated nuclear CREB1 as compared with reactive mesothelial hyperplasias and normal lung tissue. Significantly increased phosphorylated CREB1 and mRNA levels of *BCL2*, *c-FOS*, *MMP9*, and *MMP13* were also observed in MM cells *in vitro*, which were further augmented after addition of Doxorubicin (Dox). Small interfering CREB inhibited migration of MMs, increased apoptosis by Dox, and decreased *BCL2* and *BCL-xL* expression, suggesting a role for these molecules in CREB-induced MM survival. These data indicate that CREB1 and its target genes are up-regulated in asbestos-exposed human mesothelial cells through an epidermal growth factor receptor/protein kinase A pathway. Since activated CREB1

also is increased endogenously in human MM and modifies migration and resistance to Dox-induced apoptosis, inhibition of CREB1 may be a new strategy for MM therapy. (Am J Pathol 2009, 175:2197–2206; DOI: 10.2353/ajpath.2009.090400)

Malignant mesotheliomas (MMs) are derived from the mesothelial cells of the pleural, peritoneal, or pericardial cavities. Exposure to asbestos is a major risk factor for MM as ~80% of MM patients have known exposure to asbestos.^{1–3} MMs are increasing worldwide, and most patients survive <12 months after initial diagnosis.^{1–4} Thus, effective therapeutic strategies for MM are desperately needed.

cAMP response element binding protein (CREB1 or CREB) is a 43-kDa basic/leucine zipper transcription factor that regulates gene expression through activation of cAMP-dependent or -independent signal transduction pathways. CREB1 binds to an octanucleotide cAMP CRE consensus sequence in promoters of target genes as a homodimer or heterodimer with other members of the CREB/ATF superfamily. Phosphorylation of CREB1 at Ser-133 is essential for CREB-mediated transcription.⁵ Ser-133 phosphorylation promotes target gene activation in part through recruitment of the coactivator paralogs, CREB-binding protein and p300.⁶ Recruitment of CREB-binding protein by phospho-CREB1 (pCREB1) appears sufficient for CREB-mediated gene activation.^{7,8} The transcriptional coactivator pCREB-binding protein/p300 is also a histone acetyltransferase that regulates gene expression by acetylating histones and other transcription factors.

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CREB has been classically studied in the physiology of nerve or contractile cells and most recently in some cancers.^{9–13} Signaling cascades responsible for CREB activation by extracellular stimuli include protein kinase A (PKA), protein kinase C (PKC), Ca²⁺/calmodulin-dependent kinase (CaM kinases), p90 ribosomal S6 kinase, and extracellular signal-regulated kinases (ERK1/2).^{14,15} Since both PKC and ERK1/2 have been linked to cell proliferation, fibrogenesis, and mesothelial cell transformation by asbestos,^{16–19} we hypothesized that activated CREB was critical to the development and chemoresistance of MMs. Here, we first explored signaling pathways leading to phosphorylation of CREB1 and functional ramifications of silencing CREB in human mesothelial cells exposed to asbestos. We then studied activation and function of CREB in human MM cells *in vitro* in response to Dox/Adriamycin, a drug used in single-agent trials²⁰ and in a recent phase III study with Onconase.⁴ We demonstrate that crocidolite asbestos, the most potent asbestos type in the causation of MM,^{1–3} causes CREB activation in human mesothelial cells via EGF receptor (EGFR) and PKA-dependent pathways. Moreover, we show that human MM cell lines and human MM tissue arrays show high endogenous activation of CREB1 that is further increased by Dox. Silencing of CREB in asbestos-exposed human mesothelial cells or Dox-treated MMs by transfection of small interfering CREB renders them more sensitive to asbestos- or Dox-induced apoptosis. Data show roles of CREB in the development, migration, and chemoresistance of MMs.

Materials and Methods

Cell Culture and Exposure to Agents

Human peritoneal mesothelial LP9/TERT-1 (LP9) cells, an hTERT-immortalized cell line phenotypically and functionally resembling normal human mesothelial cells,²¹ were obtained from Dr. J. Rheinwald (Brigham and Women's Hospital, Harvard University, Boston, MA). This cell line was used to examine effects of asbestos on CREB activation, CREB-related gene expression, and apoptosis by asbestos. Sarcomatous (Mont) and epithelioid (Me26) human pleural MM cell lines were obtained from Drs. L. Mutti (Maugeri Foundation, Pavia, Italy) and M. Bocchetta (Loyola University, Mayfield, IL), respectively. NYU474 pleural mesothelial cells, Gard and Hmeso MM lines were contributed by Drs. H. I. Pass (New York University, New York, NY) and J. Testa (Fox Chase Cancer Center, Philadelphia, PA), respectively. Hmeso cells, originally designated H-MESO-1, were isolated by Reale et al.²² All cells were incubated at 37°C and 5% CO₂ and grown to ~80 to 90% confluency in complete medium consisting of Dulbecco's modified Eagle's medium/F12 50/50 and 10% fetal bovine serum (Mediatech, Herndon, VA), 0.1 µg/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO), 2.5 µg/ml insulin, 2.5 µg/ml transferrin, 2.5 ng/ml sodium selenite (Sigma-Aldrich), and penicillin-streptomycin (50 U/ml penicillin G and 50 µg/ml streptomycin sulfate) (Invitrogen, Carlsbad, CA).

The physical and chemical characterization of the National Institute on Environmental Health Sciences reference sample of crocidolite asbestos has been reported previously.²³ After sterilization under UV light overnight, particulates were suspended in HBSS at 1 mg/ml, sonicated for 15 minutes in a water bath sonicator, and triturated five times through a 22-gauge needle. A volume of this suspension was added to cells in medium to achieve the desired final concentration of 5 µg/cm² area dish, a concentration causing apoptosis and compensatory proliferation of surrounding pleural mesothelial cells.²⁴ The EGFR inhibitor, AG1478 (10 and 20 µmol/L), the ERK1/2 inhibitor, U0126 (10 and 20 µmol/L), the general PKC inhibitor (Bisindolymaleimide I; 5 µmol/L), a PKCδ-specific inhibitor Rottlerin (5 µmol/L), and the CaM kinase II inhibitor, KN93 (30 µmol/L), or its inactive isomer, KN92 (30 µmol/L), were obtained from Calbiochem (La Jolla, CA). The PKA inhibitor, H89 (10 µmol/L), was obtained from BIOMOL (Plymouth Meeting, PA). Forskolin (10 µmol/L for 15 minutes), an activator of CREB, and Dox were purchased from Sigma-Aldrich. All inhibitors were added at effective concentrations reported previously in the literature^{19,25,26} for 1 hour before asbestos exposure. Control cultures received medium without inhibitors but with vehicle (≤1% DMSO) instead and were treated identically. All experiments were performed in triplicate or more.

Small-Interfering RNA Experiments

On-Target plus Non-Targeting small-interfering RNA (siRNA) number 1 (scrambled control) and On-Target plus SMARTpool human CREB siRNA (100 nmol/L, Dharmacon, Lafayette, CO) were transfected into 95% confluent cells using Lipofectamine 2000 (Invitrogen), following the manufacturer's protocol. The efficiency of CREB protein knockdown was determined by quantitative RT-PCR and Western blot analysis after 24, 48, and 72 hours.

Western Blots

Cells were exposed to agents as described above, the medium aspirated, and cells washed twice with ice-cold PBS before collection in 4× sample buffer (200 µmol/L Tris (pH 6.8), 4% SDS, 4 mg/ml β-mercaptoethanol, 40% glycerol, and 2 µmol/L pyronin-Y). The amount of protein was determined using the RC DC protein assay (Bio-Rad, Hercules, CA). A total of 60 µg protein were separated by 10% SDS-PAGE and transferred to nitrocellulose. Western blots were performed as previously described,¹⁹ using antibodies specific to total and phosphorylated CREB (rabbit polyclonal anti-CREB, 1/1000, and rabbit polyclonal anti-phospho-Ser 133-CREB1/500; Cell Signaling Technology, Danvers, MA) and total β-actin 1/2000 (Abcam, Cambridge, MA). QuantityOne was used to quantify band density, and phosphorylated protein levels were normalized to respective total protein levels, ie, pCREB/CREB. Blots are representative of at least three different experiments.

Real-Time Quantitative PCR

Total RNA was prepared using an RNeasy plus mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA) as described previously.¹⁹ Total RNA (1 μ g) was reverse-transcribed with random primers using the Promega AMV Reverse Transcriptase kit (Promega, Madison, WI) according to the recommendations of the manufacturer. To quantify gene expression, the cDNA was amplified by TaqMan Real Time Q-PCR using the 7900HT Sequence Prism Detector (Applied Biosystems, Foster City, CA). Triplicate assays were performed with RNA samples isolated from at least three independent experiments. Fold changes in gene expression were calculated using the δ -delta Ct method. The values obtained from cDNAs and hypoxanthine phosphoribosyl transferase (*HPRT*) controls provided relative gene expression levels for the gene locus investigated. The Assay on Demand primers and probes used were purchased from Applied Biosystems.

Detection and Quantitation of Apoptosis

To determine whether modulation of CREB levels altered cell death in LP9 human mesothelial and MM cells, detection of apoptosis was performed using the Apostain technique as described previously.²⁷ Briefly, cells grown on glass coverslips were transfected with siCREB or scrambled control. After exposure to either asbestos or Dox (25 μ mol/L) for 24 hours, coverslips were processed to determine the numbers of apoptotic cells and total cell numbers per field. Five random fields were evaluated at $\times 400$ magnification on each coverslip.

Migration Assay

Migration was assessed using 6-well Transwell polycarbonate filters (Corning Costar, Corning, NY) with an 8- μ m pore size. Approximately 2.5×10^4 cells transfected with either siCREB or control siRNA (siC) were seeded in the upper chamber of the Transwell inserts and incubated for 72 hours at 37°C in serum-free medium. Dulbecco's modified Eagle's medium/F12 containing 10% FBS was used as a chemoattractant in the bottom chamber. Cells that did not migrate through the pores of the Transwell inserts were manually removed with a cotton swab. Cells that migrated to the bottom of the membrane were fixed in cold methanol for 10 minutes and then stained with 0.01% crystal violet in 20% ethanol. After incubating for 10 minutes, filters were washed thoroughly in water and suspended in 200 μ l of 5% acetic acid and 5% methanol. Colorimetric readings were taken at OD₅₉₅.

Immunohistochemistry

Three MM tissue arrays (obtained from Dr. H. I. Pass) were examined. Each array contained 10 to 15 MM sections from different patients with pleural mesothelioma ($N = 40$ total), 1 section of lung carcinoma (epithelioid clear cell carcinoma) and 1 section of normal lung, kidney, and liver. In addition, we evaluated three reactive mesothelial hyperplasias and four additional sections from normal lungs. In brief, slides containing 5- μ m-thick sections were deparaffinized in xylene and ethanol. After antigen retrieval at 95°C in DakoCytomation (Carpinteria, CA) target retrieval solution, sections were blocked with

Figure 1. Asbestos activates CREB in human mesothelial cells via EGFR and PKA-dependent pathways. LP9 human mesothelial cells (**A**) and NYU474-isolated human mesothelial cells (**B**) at confluency were exposed to asbestos (5 μ g/cm² dish) for the indicated time points. From cell lysates, 60 μ g protein was immunoblotted for pCREB and total CREB using specific antibodies. **C:** LP9 cells were pretreated with the PKA inhibitor, H89 (10 μ mol/L), or the ERK1/2 inhibitor, U0126 (10 μ mol/L), for 1 hour before exposure to asbestos for 4 hours. **D:** Cells were pretreated with the EGFR inhibitor AG1478 (10 and 20 μ mol/L) for 1 hour before asbestos exposure for 4 hours. * $P < 0.05$ as compared with vehicle treated control; ** $P < 0.05$ as compared with asbestos group.

“peroxidase block” and then with “protein block” sequentially for 30 minutes in a humidified chamber. After washing in PBS, sections were incubated with 1/100 dilution of polyclonal anti-rabbit pCREB (Cell Signaling Technology) overnight at 4°C in a humidified chamber. After washing in PBS, biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) was applied to sections for 1 hour at room temperature. Each section was then treated with streptavidin-horseradish peroxidase for 30 minutes in a humidified chamber, and color was developed using DAB chromogen for 5 minutes. Each slide was counterstained with Gill's hematoxylin. After brief washes in 100% ethanol and xylene, slides were air dried, and coverslips were mounted with Permaslip. For the negative control, one slide was stained as described above but without primary antibody. Slides were examined by a board certified pathologist (Dr. M. W. Bosenberg) using a blind coding system for the localization and intensity of pCREB expression (0 = no staining; 1 = weak; 2 = moderate; and 3 = strong).

Statistical Analyses

For all *in vitro* experiments, at least three independent experiments were performed ($N = 2$ to 4 samples/group/experiment). Statistical significance was evaluated by analysis of variance using the Student Neuman-Keul's procedure for adjustment of multiple pairwise comparisons between treatment groups or the nonparametric Kruskal-Wallis, Mann-Whitney, or Tukey honestly significant difference tests. Values of $P < 0.05$ were considered statistically significant.

Results

Asbestos Causes Protracted Activation of CREB in Human Mesothelial Cells through EGFR and PKA-Dependent Pathways

Since asbestos activates a number of protein kinase cascades in lung epithelial cells and mesothelial cells after phosphorylation of the EGFR,^{19,27–31} we hypothesized that one or more of these pathways might be linked to CREB1 phosphorylation by asbestos in human mesothelial cells. In both LP9 cells (Figure 1A) and freshly isolated human mesothelial cells (NYU474) (Figure 1B), asbestos ($5 \mu\text{g}/\text{cm}^2$ dish) caused increases ($P < 0.05$) in CREB1 phosphorylation that persisted for 24 hours. The positive control, Forskolin ($10 \mu\text{mol}/\text{L}$; 15 minutes), also phosphorylated CREB1 and ATF1 in human mesothelial cells (Figure 1, A and B). After addition of asbestos, more protracted phosphorylation of proteins over time may be related to the fact that asbestos fibers are insoluble and slowly precipitate on or remain within mesothelial cells.

To assess the mechanisms of activation of CREB1 by asbestos in LP9 cells, we used small-molecule inhibitors known to regulate CREB activation in other cell types.^{12,19,26–31} These included specific small-molecule inhibitors of ERK1/2 (U0126; 10 and $20 \mu\text{mol}/\text{L}$), PKA (H89, $10 \mu\text{mol}/\text{L}$), CaM kinase II (KN93 and its inactive analog, KN92; $30 \mu\text{mol}/\text{L}$), general PKCs (Bisindolymaleimide I; $5 \mu\text{mol}/\text{L}$), PKC δ (Rottlerin; $5 \mu\text{mol}/\text{L}$), and EGFR phosphorylation (AG1478; 10 and $20 \mu\text{mol}/\text{L}$) at nontoxic concentrations. As shown in Figure 1C, the PKA inhibitor, H89, completely abolished the increased CREB1 phosphorylation by asbestos, whereas the MEK1/2 inhibitor U0126 had no effect. In contrast, the EGFR inhibitor, AG1478, blocked asbestos-

Figure 2. Asbestos upregulates CREB target genes in human mesothelial cells. LP9 cells were exposed to asbestos ($5 \mu\text{g}/\text{cm}^2$ dish) for time points from 4 to 24 hours. RNA and cDNA were prepared as described in *Materials and Methods*, and quantitative RT-PCR was performed using specific primers and probe mixtures for each gene. Graphs show fold changes in steady-state levels of gene expression. * $P < 0.05$ as compared with untreated control group (0).

induced CREB activation significantly at both concentrations (10 and 20 $\mu\text{mol/L}$) (Figure 1D). Inhibitors of CaM kinase II, general PKCs, and PKC δ had no effects on asbestos-induced CREB1 activation (data not shown). These results show that asbestos-induced CREB activation involves signaling via the EGFR and PKA. It should be noted that the pCREB antibody used here also reacted with pATF1, another CREB family member (shown just below the pCREB band in Western blots).

CREB Target Genes Are Up-Regulated by Asbestos in Human Mesothelial Cells

To study whether expression of CREB-regulated genes (*BCL2*, *cFOS*, *MKP1*, *EGR1*, and *MMP2*, *MMP9*, and *MMP13*) was increased in LP9 mesothelial cells exposed to asbestos, RNA was prepared and reverse-transcribed as described in *Materials and Methods*. We chose to examine gene expression related to regulation of "early response" cell signaling (*cFOS*, *EGR1*, and *MKP1*), apoptosis (*BCL2*), and extracellular matrix (*MMP2*, *MMP9*, and *MMP13*), responses linked to asbestos after *in vitro* exposures and inhalation.^{19,25–29} As shown in Figure 2, A–C, asbestos caused significant increases in *cFOS*, *EGR-1*, and *MKP-1* expression at all time points (4, 8, and 24 hours). Significantly increased levels of *BCL2* and *MMP13* were observed at 24 hours. An unexplained decrease in *BCL2* levels also was observed at 8 hours. In contrast, mRNA levels of *MMP2* and *MMP9* did not change significantly after exposure to asbestos at any time point (data not shown). Thus, asbestos-induced CREB activation may cause up-regulation of critical CREB-regulated genes or proteins in human mesothelial cells, which have functional roles in asbestos-induced responses.

Asbestos Causes Apoptosis in Human Mesothelial Cells That Is Increased after Transfection with siCREB

We next focused on whether CREB was causally linked to apoptosis by asbestos. In addition to killing cells, asbestos-induced apoptosis also triggers compensatory proliferation of surrounding mesothelial cells²⁴ that may be linked to repair from injury and/or selective advantage of a chromosomally altered mesothelial cell population. Figure 3A shows complete knockdown of CREB protein in LP9 mesothelial cells transfected with siCREB, whereas cells transfected with nontarget control (siC) had unaltered CREB levels in comparison with untransfected cells. Exposure of siC-transfected LP9 cells to asbestos (5 $\mu\text{g}/\text{cm}^2$) for 24 hours resulted in ~28% of cells exhibiting apoptosis (Figure 3, B and C), whereas 44% of siCREB-transfected LP9 cells were apoptotic ($P < 0.05$). These data show that CREB renders human mesothelial cells more resistant to apoptosis by asbestos and may in conjunction with other signaling pathways (activated by asbestos) act in the development of MM.

Figure 3. Asbestos-induced apoptosis in human mesothelial cells is increased after transfection of siCREB. LP9 cells were grown on coverslips and were transfected with either nontargeting siC, or On-Target plus SMART pool siCREB RNA. Untransfected (UT) LP9 cells were also run in parallel. Cells were exposed to asbestos (5 $\mu\text{g}/\text{cm}^2$) for 24 hours, and apoptosis was assessed using the Apostain technique. **A:** On-Target plus siCREB attenuated CREB protein levels in LP9 cells, whereas transfection of an siC had no effect on CREB protein levels. **B:** Immunocytochemistry showing asbestos-induced apoptosis (right panels), which was further increased by siCREB in LP9 cells. Nuclei of apoptotic cells show brown staining. **Arrows** indicate asbestos fibers. **C:** Quantitation of apoptosis. * $P < 0.05$ from respective controls; ** $P < 0.05$ as compared with asbestos-exposed siRNA control-transfected cells; $\times 400$; Scale bar = 50 microns.

MM Cells Show High Endogenous pCREB Levels That Are Further Increased after Treatment with Dox

To determine whether MM cells expressed higher levels of CREB than nontransformed mesothelial cells, pCREB and CREB were measured by Western blot analyses in

Figure 4. Human MM cells show high endogenous levels of pCREB and CREB gene expression. Surgically resected human mesothelial cells (NYU474), LP9 human mesothelial cells, and five human MM cell lines were grown to confluency before preparation of cell lysates and Western blot analyses. **A:** All MM cell lines showed increased endogenous phosphorylation of CREB as compared with NYU474 and LP9 mesothelial cells. **B:** Two mesothelioma cell lines, Mont and Me26, were treated with multiple concentrations of Dox for 24 or 48 hours. * $P < 0.05$ as compared with respective untreated control. A significant increase with dose was also observed. **C:** Mont and Me26 MM lines show altered mRNA levels of CREB target genes as compared with LP9 mesothelial cells. * $P < 0.05$ as compared with LP9 group.

different MM cell lines in comparison with LP9 cells and isolated normal human mesothelial cells (NYU474). As shown in Figure 4A, all five MM lines showed increased endogenous CREB activation as compared with untransformed human mesothelial cells. Endogenous activation of CREB in MM lines could not be blocked by various inhibitors (discussed in Figure 1) even at higher concentrations (data not shown). These results prompted us to study possible roles of CREB in function and/or chemoresistance of MM cells by using siRNA approaches to inhibit CREB. For these studies, we first selected one sarcomatoid line (Mont) and one epithelioid line (Me26) to determine whether addition of Dox-altered levels of phosphorylated CREB. Treatment of these MM cell lines with Dox at different doses (0.1 to 10 μ mol/L) and time points (24 and 48 hours) showed increased dose- and time-related phosphorylation of CREB (Figure 4B).

CREB Target Genes Are Expressed Differentially in Human MM Cells in Comparison with LP9 Mesothelial Cells

We then studied endogeneous expression of selected CREB-regulated genes in Mont and Me26 MMs. In comparative experiments, confluent cell cultures were used to control for possible cell cycle effects. As shown in Figure 4C, mRNA levels of *cFOS* were significantly up-regulated in both Me26 and Mont lines. Expression of the

antiapoptotic gene *BCL2* as well as *MMP9* and *MMP13*, matrix metalloproteases involved in the degradation of extracellular matrix molecules, tumor invasiveness, and cell migration, was also highly expressed ($P < 0.05$) in both MM cells lines as compared with LP9 mesothelial cells. In contrast, *MKP1*, which dephosphorylates mitogen-activated protein kinase, was less expressed in both MM lines.

Dox-Induced Apoptosis in Human MM Cells Is Enhanced by Transfection with siCREB

To determine whether siCREB transfection modified Dox-induced apoptosis in MM cells, both Mont and Me26 lines were transfected with siC or siCREB. In Mont cells, ~56% inhibition of CREB levels occurred using this approach, whereas in Me26 cells, CREB inhibition of ~80% was achieved (Figure 5A). Me26 and Mont cells then were treated with Dox (25 μ mol/L) for 24 hours, and apoptosis was assessed using the Apostain technique, as described above. Although baseline levels of apoptosis were not affected in siCREB-transfected cells, transfection with siCREB significantly increased the percentage of apoptotic cells in both MM cell lines (Figure 5B). These data show a novel role of CREB in rendering MM cells resistant to Dox-induced apoptosis.

Figure 5. Transfection of siCREB results in increased Doxorubicin-induced apoptosis in MM cells and inhibits their migration. Mont and Me26 lines were grown on coverslips and transfected with siCREB or siC. **A:** Western blots showing effects of siCREB and siC transfection on CREB protein levels in Mont and Me26 cells at 48 hours posttransfection. β -actin levels were assessed to affirm equivalent loading of lanes. **B:** Cells were transfected with siCREB or nontargeting siC and were treated with Dox (25 μ mol/L) for 24 hours. Apoptosis was assessed using the Apostain technique as described in *Materials and Methods*. **C:** Two aggressively migrating MM cell lines (Mont and H-meso) were transfected with siCREB or nontargeting siC and assessed for migration using Transwell inserts. * P < 0.05 as compared with respective siC control group; ** P < 0.05 as compared with siCon+Dox group. siCREB inhibited *BCL2* and *BCL-xL* (C) and siCREB (D) inhibited *MMP9* genes. * P < 0.05 as compared with siC.

Migration of Human MM Cells Is Inhibited after Transfection with siCREB

Migration of MM cells is essential to their encapsulation, invasion, and growth in the pleural and peritoneal cavities. Since the epithelioid Me26 line did not test positively in a migration assay *in vitro*, we studied migration of Mont and Hmeso, a biphasic or epithelioid MM, exhibiting migration in this assay. As shown in Figure 5B, transfection with siCREB decreased migration of Mont cells by ~35% (P < 0.05). Similar trends were observed in siCREB-transfected Hmeso cells.

siCREB Inhibits Prosurvival and Migration-Related Genes in MMs

To understand the mechanisms of CREB in apoptosis and migration of MM cells, we studied four CREB regulated survival genes, *IAP-1*, *IAP-2*,³² *BCL2*, and *BCL-xL*,^{9–13} and the migration related gene, *MMP9*. Mont and Me26 MMs were transfected with siCREB or siC as described above. Inhibition of CREB significantly inhibited expression of the prosurvival gene, *BCL2*, in a time-dependent manner; however, *BCL-xL* showed a significant but transient decrease in expression at 24 hours only in both cell lines (Figure 5C). In contrast, *IAP-1* and *IAP-2* mRNA levels remained unchanged (data not shown). Our data suggest that *BCL2* and *BCL-xL* inhibition by siCREB may in part be contributing to increased apoptosis seen in these cell lines. However, the roles of other CREB-regulated genes in this process remain to be explored. CREB inhibition also inhibited *MMP9* expression in Mont cells (Figure 5D).

Tissue Arrays of Human MMs Show Increased Nuclear pCREB

To show activation of pCREB in human MM cells, we evaluated MM tissue arrays. Each array included 10 to 15 sections from the tumors of individual MM patients, 1 section of normal lung, liver, and kidney tissue and a section of lung adenocarcinoma from another patient (two of three arrays). We evaluated 33 MM sections from individual patients, 7 normal lung sections and 3 reactive mesothelial hyperplasias. Figure 6 shows representative sections from all groups. As shown in Figure 6A, representative MMs stained positively for cytoplasmic and nuclear pCREB. Normal liver and kidney sections were negative for pCREB immunoreactivity as was MM tissue in the absence of a primary antibody (data not shown). Lung tumors showed pCREB localization in the cytoplasm of one tumor and in both cytoplasm and nucleus of another tumor (data not shown), whereas a representative normal lung section (Figure 6C) showed occasional positive staining for pCREB in alveolar type II epithelial cells. Reactive mesothelial hyperplasias showed weak pCREB staining (Figure 6B). CREB can be phosphorylated in the cytoplasm and nucleus, but nuclear pCREB is the transcriptionally active form.^{7,8} Thus, both cytoplasmic and nuclear pCREB were evaluated in each MM section using a blind coding system by a board certified pathologist (M. W. Bosenberg) (Table 1). These data showed that nuclear (activated) pCREB was most predominant in MM (~75% of tissues). Thus, these *in vivo* data support our *in vitro* data that MMs have high endogenous levels of activated CREB.

Discussion

Our studies demonstrate that activation of CREB is an important transcription factor in responses of human mesothelial cells to asbestos and in human MMs treated with

Figure 6. Immunostaining of human MM tissue arrays show predominantly nuclear staining for pCREB. **A:** Two representative MM tissue sections (from a total of 33 MM sections) show strong immunoreactivity (brown) for pCREB in contrast to reactive mesothelial hyperplasia and normal lung. Negative controls consisted of MMs stained without primary antibody (data not shown). A reactive mesothelial hyperplasia (benign pleural lesion) with occasional pCREB-positive nuclei (**arrow, B**) and type II lung epithelial cells in normal lung section showed some positive pCREB staining (**arrow, C**). $\times 400$; Scale bar = 50 microns.

Dox. Here, we show that crocidolite asbestos, a potent mesotheliomagenic asbestos fiber associated with generation of oxidative stress,²⁷ causes protracted activation of CREB in human mesothelial cells via EGFR- and PKA-dependent pathways. Phosphorylation of CREB by asbestos may occur via H_2O_2 ,²⁷ since we have recently shown that inhibition of EGFR phosphorylation decreases both H_2O_2 -induced CREB phosphorylation and nuclear translocation of PKA.³³ Moreover, cross-talk between PKA and the EGFR was recently demonstrated in transgenic mice.³⁴ In addition to blocking prosurvival pathways induced by asbestos, CREB inhibition alone or in combination with inhibitors of EGFR phosphorylation may be necessary to curtail chemoresistance of MM, especially since EGFR expression and activation occur in only 60% of human MMs, and Iressa, an inhibitor of EGFR phosphorylation, has been used unsuccessfully in single modality clinical trials.³⁵ We previously demonstrated activation of CREB by asbestos in murine lung epithelial

cells via EGFR, PKA, and ERK1/2 cascades.³¹ However, in human mesothelial cells, ERK1/2, CaM kinase II, and PKC inhibitors had no effect on asbestos-induced CREB activation, suggesting that CREB signaling may be cell type- and/or species-dependent. Our findings here show that CREB activation by asbestos either alone or in conjunction with other signaling pathways activated by asbestos may augment the development of mesothelioma.

Various MM cells and tumor tissue arrays also showed endogenous activation of CREB. However, an exhaustive effort to block CREB activation by using different small molecule inhibitors (against EGFR, PKA, mitogen-activated protein kinase, PKCs, and CaM kinase II) in MM cells was not effective (data not shown). One possible explanation for these results could be that these pathways are not involved in CREB activation in MM cells as opposed to normal mesothelial cells. Alternatively, endogenously activated CREB in MM cells might be a result of constitutively inhibited protein phosphatase 1, a serine/threonine phosphatase required to inactivate CREB by dephosphorylation,³⁶ in these cells. For example, microarray data from our laboratory suggests that several human MM cell lines have significantly lower levels of protein phosphatase 1 in comparison with nonmalignant human mesothelial cells (Shukla et al, unpublished data).

We also evaluated expression of a number of CREB target genes in MM and LP9 cells in response to asbestos. Levels of *BCL2*, an antiapoptotic/survival gene transcriptionally modulated by CREB, were elevated by asbestos in mesothelial cells, an observation in line with gene expression profiling in crocidolite asbestos-exposed transformed and malignant MM cell lines where increased mRNA levels of BclII/adenovirus E1B 19-kDa interacting protein were reported previously.³⁷ Up-regulation of the BclII survival pathway by asbestos is one of

Table 1. Grading of pCREB Intensity in Human MM Tissue Arrays

Score [†]	No. of array sections given corresponding score MM*	
	Cytoplasmic	Nuclear
0	26	9
1	3	10
2	3	7
3	1	7
Positive/Total	7/33 (21.21%)	24/33 (72.72%)

*A total of 40 MMs including 18 epithelioid, 13 biphasic, and 9 sarcomatoid MMs were evaluated by histology (courtesy of Dr. M. Carbone, a board certified pathologist) of which 33 could be used and graded for pCREB localization.

[†]Intensity of staining: 0 = no staining, 1 = weak, 2 = moderate, 3 = strong.

several survival pathways reported in mesothelial cells exposed to asbestos.^{38–40}

Our data also show that MMs have endogenously up-regulated *BCL2* in comparison with LP9 human mesothelial cells. In support of our findings, it has recently been reported that MMs overexpress Bcl-x_L, another antiapoptotic member of the BclII family.⁴¹ Moreover, small molecule BclII/x_L inhibitors alone or in combination with other chemotherapeutic drugs induce apoptosis in MMs. Our studies suggest that increased Dox-induced apoptosis by siCREB may be attributed in part to decreased expression of the CREB-regulated^{9–13} prosurvival genes, *BCL2* and *BCL-xL*. However, the role of other important genes in this process cannot be excluded.

In support of our data on the importance of CREB in MM cell migration (Figure 5B), mRNA levels of *MMP9* and *MMP13*, both transcriptionally regulated by CREB and critical to cell migration, were increased severalfold in both MM cell lines as compared with LP9 mesothelial cells. In addition, we show that siCREB inhibits *MMP9* mRNA levels (Figure 5D), suggesting a possible link between activation of *MMP9* and MM migration. Increases in *MMP9* protein and activity occur after infection of primary human mesothelial cells with SV40 and exposure to crocidolite asbestos, also involving an ERK1/2/activator protein 1 pathway.⁴² In studies here, expression of *MMP13* was also increased by asbestos in LP9 cells exposed to asbestos, an event linked to transcriptional activation of *MMP13* via phosphorylation of the EGFR and ERK1/2 pathway as well as activation of PKC δ in asbestos-exposed lung epithelial cells.¹⁹

In contrast to other CREB-related genes, *MKP1* expression was significantly attenuated in MM lines in contrast to LP9 mesothelial cells that demonstrated increases in *MKP1* expression in response to asbestos (Figure 2). Since this enzyme causes dephosphorylation of activated mitogen-activated protein kinase proteins, its decreased endogenous expression in MMs may explain why phosphorylated ERK1/2 is increased in human MMs.⁴³ Increased *MKP1* mRNA levels by asbestos is puzzling in LP9 cells but may represent a compensatory response to increases in phosphorylated mitogen-activated protein kinase by asbestos in these and other cell types.^{25,28,29,31,42}

Other recent data support additional roles of activated CREB in other tumor types. For example, CREB levels are elevated in blast cells from patients with acute myeloid leukemia, resulting in abnormal proliferation and survival of myeloblast cells *in vitro* and *in vivo* via a cyclin A1 pathway.⁹ In addition, CREB is involved in hypoxia-mediated activation of the angiogenesis factor Ccn1 in lymphoma cells.⁴⁴ CREB overexpression is also critical for progression of leukemias.¹⁰ In support of these data, overexpression of CREB1 in human breast cancers correlates with poor prognosis, metastatic disease, and nodal involvement.¹¹ Moreover, others have reported constitutively activated CREB and its involvement in growth and survival of non-small cell lung cancer cells *in vitro* using ectopic expression of a dominant repressor CREB construct and siRNA approaches.¹²

In conclusion, we show for the first time that up-regulation of pCREB in MM cells by Dox is a probable mechanism of resistance to chemotherapeutic drugs. Our hypothesis is strengthened by a recent report¹³ showing that restoration of CREB-mediated transcription is important in ameliorating *cis*-platin-induced cytotoxicity in renal tubular cells *in vitro*. Our findings suggest that CREB and CREB target genes are involved not only in maintaining the migratory and invasive phenotype of aggressive MMs but also in their resistance to therapy by Dox and possibly other agents. These data provide a rationale for inhibiting CREB activity in MMs by targeted delivery of siCREB or small molecule inhibitors of CREB.

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