An Extracellular Signal-Regulated Kinase 2 Survival Pathway Mediates Resistance of Human Mesothelioma Cells to Asbestos-Induced Injury

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We hypothesized that normal human mesothelial cells acquire resistance to asbestos-induced toxicity via induction of one or more epidermal growth factor receptor (EGFR)-linked survival pathways (phosphoinositol-3-kinase/AKT/mammalian target of rapamycin and extracellular signal-regulated kinase [ERK] 1/2) during simian virus 40 (SV40) transformation and carcinogenesis. Both isolated HKNM-2 mesothelial cells and a telomerase-immortalized mesothelial line (LP9/TERT-1) were more sensitive to crocidolite asbestos toxicity than an SV40 Tag-immortalized mesothelial line (MET5A) and malignant mesothelioma cell lines (HMESO and PPM Mill). Whereas increases in phosphorylation of AKT (pAKT) were observed in MET5A cells in response to asbestos, LP9/TERT-1 cells exhibited dose-related decreases in pAKT levels. Pretreatment with an EGFR phosphorylation or mitogen-activated protein kinase kinase 1/2 inhibitor abrogated asbestos-induced phosphorylated ERK (pERK) 1/2 levels in both LP9/TERT-1 and MET5A cells as well as increases in pAKT levels in MET5A cells. Transient transfection of small interfering RNAs targeting ERK1, ERK2, or AKT revealed that ERK1/2 pathways were involved in cell death by asbestos in both cell lines. Asbestos-resistant HMESO or PPM Mill cells with high endogenous levels of ERKs or AKT did not show dose-responsive increases in pERK1/ERK1, pERK2/ERK2, or pAKT/AKT levels by asbestos. However, small hairpin ERK2 stable cell lines created from both malignant mesothelioma lines were more sensitive to asbestos toxicity than shERK1 and shControl lines, and exhibited unique, tumor-specific changes in endogenous cell death-related gene expression. Our results suggest that EGFR phosphorylation is causally linked to pERK and pAKT activation by asbestos in normal and SV40 Tag-immortalized human mesothelial cells. They also indicate that ERK2 plays a role in modulating asbestos toxicity by regulating genes critical to cell injury and survival that are differentially expressed in human mesotheliomas.

Keywords: mesothelioma; asbestos; toxicity; epidermal growth factor receptor; protein kinase B/AKT

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Exposure to crocidolite asbestos fibers is associated with the development of malignant mesothelioma (MM), a fatal tumor arising from the mesothelial cells of the pleura, peritoneum, and occasionally the pericardium (1–3). The molecular events that precede the development of MM are unclear, but activation of signaling pathways, including extracellular signal-regulated kinases (ERKs) 1/2 (4–6) and phosphoinositol-3-kinase (PI3K)/protein kinase B (AKT) signaling pathways (7) have been linked to injury by asbestos, and may be increased endogenously in some human MMs (8–10). Although at least eight members of the ERK family exist, ERK1 and ERK2, the predominant forms in mammalian cells, have been the most widely studied. Additionally, they are most often studied in tandem, because commercial antibodies for histochemistry and synthetic inhibitors fail to discriminate between ERK1 and ERK2. Many studies suggest that both ERK1 and ERK2 compete for upstream mitogen-activated protein kinase kinases (MEK1/2) and other substrates (11). Moreover, the intensity and duration of ERK signaling, intracellular compartmentalization of phosphorylated (activated) ERKs (12), substrate specificity, and downstream consequences of ERK signaling, including activation of selected transcription factors, may determine phenotypic and functional cell outcomes (13, 14). Activation of these cascades by asbestos fibers may play a role in initial cell injury, as well as compensatory mesothelial cell proliferation that favors survival of genetically altered cells in carcinogenesis.

Studies from our laboratories have shown that crocidolite asbestos fibers interact with the external domain of the epidermal growth factor (EGF) receptor (EGFR) to cause dimerization, activation, and increased EGFR mRNA and protein levels in rat (4, 6, 15, 16) and human simian virus 40 (SV40)-immortalized mesothelial (MET5A) cells (17). Moreover, phosphorylation of the EGFR by asbestos is linked to phosphorylation of ERK1 and ERK2 (ERK1/2) in normal rodent mesothelial cells (4). Likewise, AKT is regulated, in part, by PI3K, which, in turn, may be activated by phosphorylation of the EGFR in some cell types (18, 19).

SV40 has been implicated as a risk factor in MM (20, 21). Multi-institutional studies show that SV40 DNA occurs in over 60% of human MMs (22). Moreover, cooperative transforming and carcinogenic effects of SV40 and asbestos have been demonstrated *in vitro* and *in vivo* (22, 23). Both ERK1/2/activator protein-1 (23) and PI3K/AKT/mammalian target of rapamycin (mTOR) (7) survival pathways have been implicated in SV40- and asbestos-mediated mesothelial cell transformation, but how these pathways are initiated at the cell surface by asbestos, and their respective downstream effects, are poorly understood. Moreover, it is unclear whether human mesothelial cells are more sensitive to asbestos than SV40-infected or MM cells, and whether or not differential sensitivity to the toxic effects of asbestos reflects the induction of different survival pathways in these cell types. Because the induction of the ERK1/2 and PI3K/AKT/mTOR survival

pathways are problematic in chemoresistance of MMs and a variety of other tumors (9, 10, 24), we hypothesized that these pathways might also be important in prevention of acute asbestos toxicity and/or carcinogenicity. To address these questions, we first evaluated two normal lines (HKNM-2, LP9/TERT-1 or LP9), one SV40 Tag-immortalized line (MET5A), and two MM lines (HMESO, an epithelioid MM, and PPM Mill, a sarcomatoid MM) for toxicity over a range of crocidolite asbestos fiber concentrations. We then evaluated patterns of activation of PI3K/AKT and ERK1/2 pathways by asbestos in these lines and effects of small molecule inhibitors of EGFR phosphorylation, ERK1/2 phosphorylation, and PI3K on modulation of these pathways. Because asbestos exposures did not cause phosphorylation of the ERK1/2 pathway in asbestos-resistant MM lines, we dissected the effects of individual ERK1 and ERK2 kinases on asbestos resistance using RNA interference approaches. These studies showed that small hairpin ERK2 (shERK2) stable MM lines, as compared with shERK1- and shControl (shCon) stable lines, were most sensitive to asbestos toxicity. Moreover, we explored differences in endogenous death- and survival-related gene expression in shERK1- and shERK2 stable lines in comparison to shCon lines. Our results suggest an increasing gradation of resistance to asbestos toxicity as human mesothelial cells become tumorigenic. After exposure to crocidolite asbestos, EGFR-linked ERK1/2 and PI3K/AKT/mTOR pathways are stimulated in SV40-immortalized cells, whereas MMs appear to have high constitutive levels of these proteins that may mask further increases in activation by asbestos (8, 9). Our demonstration that blocking ERK2 increases asbestos toxicity in MMs that are highly resistant to asbestos suggests that targeting this survival pathway, either pharmacologically or genetically, may be a novel approach for preventing acute asbestos toxicity or pleural disease in high-risk individuals.

MATERIALS AND METHODS

Chemicals and Inhibitors

Chemicals were purchased from Sigma (St. Louis, MO). Small molecule inhibitors were from Calbiochem (La Jolla, CA) (4–6, 9, 10, 25).

Cell Cultures

Pleural HKNM-2 cells were isolated at autopsy by Dr. Helmut Popper (University of Graz, Graz, Austria). The LP9/TERT-1 (LP9)-immortalized mesothelial cell line, the sarcomatoid pleural MM line (PPM Mill), and the SV40 Tag-immortalized mesothelial cell line (MET5A) have been described previously (26, 27). The HMESO cell line was originally characterized by Reale and colleagues (28). All lines were maintained in Dulbecco's modified Eagle medium/F-12 medium (10% FBS) with additives (25). HKNM-2 cells were grown in Optimem/Hams F-12 3:1 containing 20% FBS, EGF (20 ng/ml), insulin (0.5 μ g/ml), hydrocortisone (0.4 μ g/ml), penicillin (50 units/ml), and streptomycin (100 μ g/ml). At confluency, 0.5% FBS-containing medium was added.

Lactate Dehydrogenase Release, Viability Assay, and Phase Contrast Microscopy

Lactate dehydrogenase (LDH) release was measured using the Cytotox 96 kit (Promega Corp., Madison, WI) per the manufacturer's recommendations. The trypan blue exclusion assay was used to assess cell viability (29).

Western Blot Analyses

Cells were exposed to crocidolite asbestos (25) with or without pre-addition for 1 hour of AG1478 (10, 20 μ M), an inhibitor of EGFR phosphorylation, LY294002 (10 or 20 μ M), a PI3K inhibitor, U0126 (10 or 20 μ M), a MEK1/2 inhibitor, or U0124 (20 μ M), an inactive analog of U0126, and Western blots were performed with β -actin as a loading control and quantitated (4–6, 9, 10).

Transient Transfection of LP9 and Met5A cells

On-Target plus Nontargeting siRNA no. 1 (scrambled control) and On-Target plus SMART pool human ERK1, ERK2, and AKT1 siRNA (100 nM; Dharmacon, Lafayette, CO) were transfected into LP9 or Met5A using Lipofectamine 2,000 (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The efficiency of target gene knock-down was determined by quantitative RT-PCR (qRT-PCR) (*see* Figure E2 in the online supplement).

Creation of shERK1- and shERK2 Stable MM Lines

Confluent HMESO and PPM Mill cells were transfected with shERK1, shERK2, or scrambled control Sure Silencing Plasmids (4 sh sequences for each gene were used) from SABiosciences (Frederick, MD), using Lipofectamine 2,000. After selection in G418-containing medium, all clones were screened by qRT-PCR for ERK mRNA levels. Selected clones from each group were processed by limited dilution to obtain clones in which individual ERKs were inhibited by more than 70% in comparison to shCon clones.

Affymetrix Gene Profiling

RNA samples from three independent experiments ($n = 3$ independent biological replicate group) were prepared and analyzed, as described previously (25). A Human U133A 2.0 array (Affymetrix, Santa Clara, CA) was used. Microarray data were analyzed using GeneSifter software (VizX Labs, Seattle, WA). A threefold cut-off limit was used for significance. qRT-PCR was performed (25) to validate microarray data. Fold changes in gene expression were calculated using the $\Delta\Delta$ Ct method using hypoxanthine phosphoribosyl transferase 1 as the normalization control. Assay-on-demand primers and probes used were purchased from Applied Biosystems (Foster City, CA).

Statistical Analyses

Data were evaluated by one-way ANOVA using the Student-Newman-Keul's procedure for adjustment of multiple comparisons.

RESULTS

Human MMs and MET5A Cells Are More Resistant to Crocidolite Asbestos-Induced Toxicity than HKNM-2- and LP9/TERT-1-Immortalized Human Mesothelial Cells

We first analyzed toxicity 24 hours after addition of crocidolite asbestos over a range of concentrations in all cell lines using LDH release, trypan blue exclusion, and phase contrast microscopy. Over a 24-hour period, both normal HKNM-2 pleural mesothelial cells (Figure 1A) and LP9 cells (Figure 1B) showed dose-related increases ($P \leq 0.05$) in LDH release that correlated with rounding up and sloughing of cells (Figure 1F) at asbestos concentrations from 5 to 25 μ g/cm² dish. In contrast to the greater than fourfold increases in LDH release observed at 20–25 μ g asbestos/cm² dish in these normal cell lines, less than twofold increases in LDH release, and no morphologic indications of toxicity, were observed in MET5A cells (Figures 1C and 1G). In HMESO MMs, significantly increased LDH release was not observed (Figure 1D), but decreases in cell viability occurred at 10 μ g asbestos/cm² dish and higher concentrations in both MET5A and HMESO cells (Figure E1). PPM Mill MM cells showed no evidence of morphologic injury, increased LDH release (Figure 1), or decreased cell viability by asbestos at concentrations as high as 20 μ g asbestos/cm² dish (Figure E1).

MET5A Cells Show Increased Phosphorylated AKT in Response to Serum Deprivation or Asbestos, whereas LP9 Cells Exhibit Decreased Phosphorylated AKT Levels

Because numbers of normal isolates of HKNM-2 human mesothelial cells were too limited for Western blot experiments, we used LP9 and MET5A cells comparatively to determine patterns of up-regulation of AKT or ERK1/2 survival pathways in response

Figure 1. Simian virus 40 (SV40) Tag-immortalized human mesothelial cells (MET5A) and human malignant mesotheliomas (MMs) (HMESO and PPM Mill) are more resistant to crocidolite asbestos-induced toxicity (National Institute of Environmental Health Sciences reference sample) than normal pleural mesothelial cells (HKNM-2) and a telomerase-immortalized peritoneal mesothelial (LP9) cell line. (A–E) Lactate dehydrogenase (LDH) release assays after exposure of cells to asbestos for 24 hours. (F and G) Phase contrast microscopy showing increased sensitivity of LP9 cells (F) versus MET5A cells (G) to asbestos (white arrows indicate long crocidolite fibers); all at 400 $\times$ magnification. All quantitative data are graphed as means ($\pm$ SEM); $n = 3$ per group in duplicate or more experiments. * $P \leq 0.05$ in comparison to no-exposure control (0) groups. Asb, asbestos.

to asbestos or serum deprivation. Moreover, we hypothesized that activation of the EGFR was a common upstream event leading to activation of both of these pathways. In these studies, we first examined levels of phosphorylated AKT (pAKT) and total AKT at periods from 4 to 24 hours after addition of asbestos and maintenance in serum-free medium (a situation known to induce pAKT in proliferating or transformed cell types over time). In addition, H_2O_2 was used as a positive control in these studies. As shown in Figure 2A and the representative Western blot in Figure 2E, addition of asbestos caused dose-related decreases in pAKT/

AKT levels in LP9 cells. In contrast, increases in pAKT/AKT levels were observed in MET5A cells after asbestos exposure (Figure 2B). pAKT/AKT levels in MET5A cells were inhibited ($P \leq 0.05$) by pretreatment with 20 μ M of the EGFR phosphorylation inhibitor, AG1478 (Figure 2C), or the PI3K inhibitor, LY204002, at 10 and 20 μ M (Figure 2D). Both of these compounds also significantly inhibited endogenous pAKT/AKT levels in MET5A cells. These results suggest that asbestos-induced pAKT/AKT levels in SV40 Tag-immortalized MET5A cells are linked to PI3K and phosphorylation of the EGFR.

Figure 2. Different patterns of AKT activation are observed in LP9 cells in contrast to SV40 Tag-immortalized human mesothelial cells (MET5A). (A–D) Quantitation of pAKT/AKT levels in LP9 and MET5A cells, maintained in serum-free medium. H₂O₂ (500 μ M for 30 min) was used as a positive control for induction of pAKT in LP9 cells (A). The epidermal growth factor receptor (EGFR) phosphorylation inhibitor, AG1478 (AG) (C), or the phosphoinositide-3-kinase (PI3K) inhibitor, LY29002 (LY) (D), were added 1 hour before asbestos. (A and B) The 12-hour control group was used for comparison with asbestos groups at 4, 6, and 12 hours, and the 24-hour control group was used for 24-hour asbestos group comparisons. Data are graphed as means ($\pm$ SEM); $n = 3$ per group in duplicate or more experiments. * $P \leq 0.05$ in comparison to no-exposure control (0) group; † $P \leq 0.05$ in comparison to asbestos-alone group. (E) A representative Western blot for LP9 cells.

Asbestos Causes Stimulation of the pERK1/2 Pathway in LP9 Cells and MET5A Cells that Is Inhibited by Preaddition of U0126 or AG1478

Asbestos-induced elevations in phosphorylated ERK (pERK) 1/2 levels in LP9 (Figures 3A and 3E) and MET5A cells (Figures 3B and 3F) were inhibited by the MEK1/2 inhibitor, U0126, at both 10 and 20 μ M. U0126 alone also diminished endogenous levels of pERK1/2 in both cell lines. These patterns were observed at 8, 12, and 24 hours. Asbestos-associated, dose-dependent increases in pERK1/2 also were abrogated after preaddition of AG1478 to both cell lines (Figures 3C and 3D).

Asbestos Does Not Induce ERK1 and ERK2 or AKT Phosphorylation in MMs after Exposure to Asbestos

To determine if increased ERK1/2 and AKT phosphorylation was also observed in MM lines, we exposed both MM lines to asbestos for 8 and 24 hours. At neither time point were pERK1, pERK2, or pAKT levels significantly increased by exposure to asbestos (Figure 4). In HMESO cells, significant decreases in

pERK1/ERK1 levels were seen in both asbestos-treated groups in comparison to unexposed cells at 24 hours, but changes were not dose related.

Transient Transfection of LP9 and MET5A Cells with siRNAs Targeting ERK1, ERK2, and AKT Affect Asbestos-Induced Viability

ERK1 and ERK2 may have redundant or opposing roles in cell injury, survival, and carcinogenesis as they compete for substrates (11). Moreover, AKT activation is a known determinant of cell fate (18, 19). We next evaluated the effects of RNA interference using transient transfection of siERKs and siAKT on modulation of asbestos-induced decreases in cell viability. The specificity of these transfections is indicated in Figure E2. Consistent with the hypothesis that ERKs are intrinsic to cell survival in both cell lines, asbestos-induced decreases in cell viability were further decreased in both siERK1- and siERK2-transfected LP9 and MET5A lines as compared with siControl-transfected cells (Figures 5A and 5B). Consistent with results in Figure 2 showing

Figure 3. Asbestos causes stimulation of the phosphorylated extracellular signal-regulated kinase (pERK) 1/2 pathway in LP9 cells (A, C, and E) and SV40-immortalized MET5A cells (B, D, and F) that is inhibited by the small molecule inhibitor of mitogen-activated protein kinase kinase (MEK) 1/2, U0126, or the EGFR phosphorylation inhibitor, AG1478. H₂O₂ (500 μ M for 30 min) was used as a positive control for pERK1/2 induction in both cell types. Data are graphed as means ($\pm$ SEM); $n = 3$ per group in duplicate or more experiments. * $P \leq 0.05$ in comparison to corresponding no-exposure control (0) groups (white bars). † $P \leq 0.05$ in comparison to asbestos-alone groups.

increased pAKT/AKT levels in MET5A cells after asbestos-induced stress, targeting the AKT pathway also resulted in increased cell injury by asbestos (i.e., decreases in viable cells) in MET5A cells (Figure 5B).

shERK2 Stable HMESO and PPM Mill Lines Exhibit Increased Toxicity to Asbestos

To determine if ERK1 or ERK2 played different roles in cell survival or resistance to asbestos in MM lines, we evaluated cell viability in shCon, shERK1-, and shERK2 stable cell lines comparatively after exposure to asbestos. These studies showed that shERK2 stable cell lines generated from both cell types were the most sensitive to asbestos ($P \leq 0.05$) (Figures 5C and 5D). In the PPM Mill line, cell viability was also significantly decreased in shERK1 cells as compared with shCon cells, but effects were not as striking as observed in shERK2 PPM Mill cells.

Gene Expression of Cell Death-Related Genes in HMESO and PPM Mill shERK Stable MM Cell Lines

To probe the patterns of gene expression related to cell death and survival in MM lines and their modulation by ERK2, Affymetrix microarrays and GeneSifter Analysis were used to elucidate endogenous gene expression alterations in shERK1 and shERK2 versus shCon cell lines. As shown in Table 1, silencing ERK1 in epithelioid HMESO MM cells caused significantly increased expression of 15 genes and decreased expression ($P \leq 0.05$) of 22 genes. In contrast, shERK1 PPM Mill sarcomatous MM cells showed increased expression of six genes and decreased expression of seven genes in comparison to

the shCon PPM Mill line (Table 1). These significant mRNA differences did not reflect common genes in different MM cell lines. Whereas the significant increases or decreases in mRNA levels of genes were generally 10-fold or less in both shERK1 MM cell lines, striking increases in neurofilament, heavy polypeptide (27-fold), and peroxiredoxin (48-fold) were noted in the shERK1 HMESO cell line.

Silencing ERK2 resulted in increased ($\geq$ threefold) expression of eight genes and decreased expression of three genes ($P \leq 0.05$) in HMESO MMs. In contrast, mRNA levels of seven genes were increased and eight genes decreased ($P \leq 0.05$) in PPM Mill cells. These gene expression changes were different in each shERK2 MM cell line, as was the pattern in shERK1 MM lines. However, as indicated by bold type in Table 1, the three mRNAs decreased in the shERK2 HMESO line were also decreased in the shERK1 HMESO line. In addition, two common genes (Retinoic acid receptor, β and Superoxide dismutase 2, mitochondrial) were increased and decreased (Growth differentiation factor 5 and Tumor necrosis factor receptor superfamily member 11b), respectively, in shERK1 and shERK2 PPM Mill lines.

DISCUSSION

Asbestos fibers are a chemically and physically diverse group of naturally occurring minerals that have been linked to the development of asbestosis, lung cancers, and MMs. Iron-containing crocidolite and amosite asbestos are considered more potent than other asbestos types in the generation of reactive oxygen species, their potential to cause oxidative DNA damage (reviewed in Ref. 30), and in the development of human MMs (1–3). More

Figure 4. Individual quantitation of pERK1/ERK1, pERK2/ERK2, and pAKT/AKT levels in HMESO (A and C) and PPM Mill (B and D) MMs after exposure to asbestos. Data are graphed as means ($\pm$ SEM); $n = 3$ per group in duplicate experiments. * $P \leq 0.05$ in comparison to no-exposure control (0) group.

recently, SV40 virus has been proposed as a cofactor or cocarcinogen in MM (7, 20–23).

In studies here, we first show that SV40-immortalized MET5A, as opposed to LP9/TERT-1-immortalized mesothelial cells or HKNM-2 isolated human mesothelial cells, exhibit increased resistance to asbestos, a phenomenon that may be linked, in part, to more efficient induction of a PI3K/AKT pathway in the SV40 Tag-immortalized cells. In contrast, LP9 cells exhibited dose-related decreases in pAKT/AKT levels. These results are in agreement with experiments showing that SV40-dependent AKT activity plays a role in mesothelial cell transformation after exposure to asbestos (7). It is difficult to extrapolate whether

concentrations of asbestos used in *in vitro* studies are comparable to *in vivo* exposures leading to tumorigenesis, but it is clear from our results that increases in pAKT are dose related and occur at concentrations below those inducing overt cytotoxicity in SV40-immortalized mesothelial cells.

Results here suggest a novel mechanism of tumor promotion, whereby SV40-induced AKT renders mesothelial cells more prone to survival and proliferation after exposure to asbestos rather than embarking upon a cytotoxicity or cell death pathway. Moreover, we show that blocking phosphorylation of the EGFR blocks asbestos-induced activation of both the PI3K/AKT in SV40-immortalized cells and the ERK1/2 survival pathways

Figure 5. RNA interference studies to inhibit ERK1, ERK2, and AKT. Transient transfection studies were performed in LP9 and MET5A cells (A and B), whereas stable small hairpin Control (shCon), shERK1, and shERK2 MM lines were created from HMESO (C) and PPM Mill (D) MMs. Cell lines were evaluated for cell viability at 24 hours using the trypan blue exclusion technique. Data are graphed as means ($\pm$ SEM); $n = 3$ per group in duplicate experiments. * $P \leq 0.05$ in comparison to no-exposure control (0) groups; † $P \leq 0.05$ in comparison to asbestos-exposed shCon group.

TABLE 1. MICROARRAY ANALYSIS OF CHANGES IN CELL DEATH-RELATED GENE EXPRESSION IN SMALL HAIRPIN EXTRACELLULAR SIGNAL-REGULATED KINASE VERSUS SMALL HAIRPIN CONTROL HUMAN MALIGNANT MESOTHELIOMAS

	Increased Expression	Decreased Expression
HMESO		
shERK1	Bradykinin receptor B2 (4) CD24 molecule (5) Growth arrest specific 1 (4) Growth hormone receptor (4) Inhibitor of DNA binding 1, dominant negative helix loop helix protein (4) Inhibitor of DNA binding 3, dominant negative helix loop helix protein (3) Msh homeobox 2 (3) Protein kinase C, beta (8) Rho guanine nucleotide exchange factor (GEF) 3 (5) Secreted frizzled-related protein 1 (5) Serpin peptidase inhibitor, clade B (ovalbumin), member 2 (4) SMAD family member 6 (4) Synuclein, alpha (non A4 component of amyloid precursor) (4) Vanin 1 (4) WW domain containing oxidoreductase (3)	Aldolase C, fructose-biphosphate (5) Brain-derived neurotrophic factor (8) Deleted in liver cancer 1 (3) Fas apoptotic inhibitory molecule (4) Folate receptor 1 (adult) (3) Galanin prepropeptide (8) Gap junction protein, alpha 1, 43kDa (8) Heat shock 22 kDa protein 8 (4) Hyaluronoglucosaminidase 1 (4) Insulin like growth factor binding protein 3 (6) Interferon, alpha inducible protein 3 (3) Matrix metalloproteinase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase) (6) Neuregulin 1 (10) Neurofilament, heavy polypeptide (27) P21 protein (Cdc42/Rac)-activated kinase 7 (3) Peroxiredoxin 2 (48) Protein kinase C, alpha (4) Protein tyrosine phosphatase, nonreceptor type 6 (3) Suppressor of cytokine signaling 2 (4) Thioredoxin interacting protein (7) Tumor necrosis factor (ligand) superfamily, member 18 (4) XIAP associated factor 1 (5) Brain-derived neurotrophic factor (4) Insulin-like growth factor binding protein 3 (6) P21 protein (Cdc42/Rac) -activated kinase 7 (11)
shERK2	Adenosine A1 receptor (3) Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase* (8) Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit) (3) Myocyte enhancer factor 2C (4) Pleckstrin homology-like domain, family A, member 1 (4) SRY (sex determining region Y)-box 9 (5) TNF receptor-associated factor-1* (3) Tumor necrosis factor (ligand) superfamily, member 10 (5)	
PPM Mill		
shERK1	BTG family, member 2 (4) Cytoplasmic FMR1 interacting protein 2 (5) Interleukin 7 receptor (4) Retinoic acid receptor, beta (5) Sodium channel, voltage gated, type II, alpha subunit (4) Superoxide dismutase 2, mitochondrial (3)	Brain expressed, associated with Nedd4 (4) Cannabinoid receptor 1 (brain) (4) Growth differentiation factor 5 (6) Killer cell lectin-like receptor subfamily K, member 1 (4) Phospholipase A2, group IVA (cytosolic, calcium dependent) (5) T-cell lymphoma invasion and metastasis 1 (5) Tumor necrosis factor receptor superfamily, member 11b (14)
shERK2	Actinin, alpha 2 (3) Fibroblast growth factor 2 (basic) (4) Heme oxygenase (decycling) 1 (3) Protein phosphatase 2 (formerly 2A), regulatory subunit B, beta isoform (4) Retinoic acid receptor, beta (5) Sequestosome 1 (3) Superoxide dismutase 2, mitochondrial (4)	Calcium/calmodulin-dependent protein kinase ID (3) Complement component 6 (5) Complement component 7 (14) Growth differentiation factor 5 (5) Protein tyrosine phosphatase, non-receptor type 6 (12) Secretogranin II (chromogranin C) (6) Tumor necrosis factor receptor superfamily, member 11b (14) V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian) (6)

Definition of abbreviation: shERK, small hairpin extracellular signal-regulated kinase.

Numbers in parentheses indicate fold increases or decreases in mRNA expression; genes presented in bold text are common between shERK1 and shERK2 in the given cell line.

* Validated by quantitative RT-PCR.

induced in both LP9- and SV40-immortalized MET5A cells. Taken together, these data suggest that the activation of the EGFR by asbestos fibers and instigation of these survival cascades may allow a population of asbestos-altered mesothelial cells to be selected and/or expanded in a potentially adverse environment, such as that associated with oxidant-generating asbestos fibers. Because asbestos fibers are known to interact with a number of other receptors, including platelet-derived growth factor receptors, which are overexpressed on MMs (31) and also lead to ERK phosphorylation, EGFR activation is probably not the only pathway activating the ERK cascades in mesothelial cells. Moreover, synthetic inhibitors used here may

not be entirely specific for prevention of EGFR phosphorylation or inactivation of PI3K.

In SV40-immortalized or transformed MM, ERK and AKT pathways may act synergistically to increase cell proliferation and parameters of tumorigenesis, such as cell migration and invasion. For example, it has been shown that the ERK1/2 and AKT pathways work cooperatively in radial growth and invasiveness of melanomas, where AKT functions as a molecular switch that increases angiogenesis and the generation of reactive oxygen species, in part by a mechanism stabilizing cells with extensive mitochondrial DNA mutations generating superoxide (32). The importance of oxidative stress-induced EGFR/AKT activation in

normal cell survival also has been suggested by others reporting that EGFR-dependent AKT activation enhances survival of keratinocytes in an H₂O₂-induced toxic environment (33). Our results showing that phosphorylation of the EGFR is linked to induction of AKT and ERK1/2 survival pathways in SV40-transformed and possibly human MM cells is supported by research showing that the human EGFR (HER2, ERB2) regulates angiopoietin-2 expression via both AKT and ERK1/2 pathways in human breast cancer cells (34).

Our results suggest a redundancy of downstream survival signaling that is more robust in SV40-immortalized and malignant mesothelial cells. One explanation may be that ERK1/2 and activator protein-1 activation are up-regulated synergistically by SV40 Tag and asbestos in mesothelial cells, a putative mechanism of cocarcinogenesis (23). Moreover, p53 and Rb, known tumor suppressor genes, are inactivated by SV40 Tag (35). SV40 Tag also induces multiple autocrine pathways in human mesothelial cells and MM cells via activation of tyrosine kinase receptors for HGF (i.e., MET) (7), insulin-like growth factor-1 (36), and vascular endothelial growth factor (37), all linked to downstream activation of ERKs or PI3K/AKT. Moreover, several tyrosine kinases, including EGFR and MET, are coactivated in a number of human MM cell lines (38).

Although Western blot analyses and proteomic screens have identified critical kinase cascades linked to cell proliferation, injury, transformation, and a variety of other functional changes in cells, little is known about the gene expression regulated by specific ERK kinases. Based upon our observations that shERK2 stable MM cell lines were most sensitive to asbestos-induced toxicity, and data showing that both siERK1 and siERK2 modulated asbestos-induced stress in LP9 and MET5A cells, we explored the constitutive expression of cell death and survival-related genes significantly up- or down-regulated in comparison to shCon cell lines (Table 1). As reported by others in epithelioid and fibroblastoid MM lines (39, 40), different patterns of endogenous gene expression were observed in epithelioid HMESO versus sarcomatoid PPM Mill cells. However, shERK1 and shERK2 cells (as compared with shCons) showed some common alterations in mRNA levels in individual cell lines. Patterns of gene expression, regardless of cell line, indicated both apoptotic and necrotic-linked genes. For example, in the shERK2 HMESO line, the increased expression of three genes linked to induction of apoptosis or necrosis were observed, including TNF receptor-associated factor-1, TNF (ligand) superfamily member 10, and caspase-1, an apoptosis-related cysteine peptidase cleaving inactive pro-IL-1 β to mature IL-1 β . Caspase-1 is also a component of the Nalp3 inflammasome, which we have linked recently to asbestos-induced oxidative stress, cytokine production, and inflammation (41).

Our gene-profiling experiments also reveal a number of increases in gene expression linked to activation of the ERK cascades in other cell types, but previously unreported in mesothelial or MM cells. For example, adenosine A1 receptors have been associated with ERK1/2 activation and secretion of matrix metalloproteinase-2 in human trabecular meshwork cells (42). Activation of the ERK1/2 pathway also causes termination of agonist signaling and priming of tumor cells for recovery and *de novo* synthesis of adenosine A1 receptors (43). Moreover, adenosine A1 receptor stimulation induces transactivation of the EGFR via a PI3K/Src kinase signaling pathway by forming an immunocomplex with EGFR, causing sustained phosphorylation of ERK1/2 (44).

Myocyte enhancer factor (MEF) 2 transcription factors historically have been studied in muscle and neuronal differentiation and fate (45), but more recently in other cell types. In vascular smooth muscle cells, inducible expression of NOX1, a major source of

production of superoxide, is governed by the activating transcription factor-1-myocyte enhancer factor 2B cascade and upstream involvement of an ERK1/2-JunB pathway (46). Moreover, ERK1/2 is required for both the transcriptional and neuroprotective activity of myocyte enhancer factor 2C (47). In addition, shERK1 and shERK2 MM cells exhibited altered expression of a number of growth factor genes, as well as genes encoding their receptors (Basic fibroblast growth factor, Retinoic acid receptor beta, Insulin-like growth factor binding protein 3), genes involved in oxidative stress (Heme oxygenase-1, SOD2), and kinases (p21-activated kinase 7, calmodulin-dependent protein kinase 1D) or phosphatases. These data suggest links between ERKs and several downstream genes important in repair and proliferation after cell injury by asbestos. Because blocking ERK2 selectively in human MMs was most effective in increasing asbestos-associated MM injury, and has recently been shown to play a critical role in curtailing growth and chemoresistance of human MM cells in a severe combined immune deficient mouse xenograft model (29, 48), ERK inhibition may be a novel therapeutic approach for patients with MM.

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