

# SV40-Dependent AKT Activity Drives Mesothelial Cell Transformation after Asbestos Exposure

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## Abstract

**Human malignant mesothelioma is an aggressive cancer generally associated with exposure to asbestos, although SV40 virus has been involved as a possible cofactor by a number of studies. Asbestos fibers induce cytotoxicity in human mesothelial cells (HMC), although cell survival activated by key signaling pathways may promote transformation. We and others previously reported that SV40 large T antigen induces autocrine loops in HMC and malignant mesothelioma cells, leading to activation of growth factor receptors. Now we show that SV40 induces cell survival via Akt activation in malignant mesothelioma and HMC cells exposed to asbestos. Consequently, prolonged exposure to asbestos fibers progressively induces transformation of SV40-positive HMC. As a model of SV40/asbestos cocarcinogenesis, we propose that malignant mesothelioma originates from a subpopulation of transformed stem cells and that Akt signaling is a novel therapeutic target to overcome malignant mesothelioma resistance to conventional therapies.** (Cancer Res 2005; 65(12): 5256-62)

## Introduction

Malignant mesothelioma is an aggressive cancer usually associated with exposure to asbestos (1). The polyomavirus SV40 has been proposed as a cocarcinogen involved in the development of some malignant mesotheliomas (2), mainly due to expression of the large T antigen (Tag), which can inactivate products of tumor suppressor genes and promote cell cycle abnormalities (3).

Apoptosis is an active physiologic mechanism regulating cell death during development and other biological processes (4). Impairment of the apoptotic response may promote the survival of DNA-damaged cells, thereby contributing to carcinogenesis (5).

Asbestos fibers may induce chromosomal aberrations (6) and promote apoptosis of mesothelial cells via fiber uptake (7) and subsequent production of oxygen-free radicals (8). SV40 Tag-positive mesothelial cells are less sensitive to asbestos, and surviving cells may exhibit extensive genomic instability (9), leading to cell transformation (10). Progressive resistance to apoptosis may represent part of a multistep process leading to neoplastic transformation of human mesothelial cells (HMC; ref. 11).

We previously showed that SV40 expression induces an Rb-dependent hepatocyte growth factor (HGF)/Met autocrine loop in malignant mesothelioma cells as well as in HMC transfected with Tag (12). Interestingly, microarray analysis revealed that expression of *Met*, among other genes, is associated with asbestos exposure in mesothelial cells (13) and several other growth factors are released by SV40-positive HMC and malignant mesothelioma cells, including insulin-like growth factor-I (IGF-I) and vascular endothelial growth factor (VEGF; refs. 14, 15).

Activation of tyrosine kinase receptors by ligands induces phosphatidylinositol-3 kinase (PI3K) and Akt activities, exerting several biological effects, including increased cell survival with relevant effects on human carcinogenesis (16, 17).

In this report, we show that HMC and malignant mesothelioma cells, exposed to asbestos fibers, or to other toxic agents, become markedly resistant to cytotoxicity, due to Tag-dependent Akt activity. These findings offer novel insights with regard to molecular mechanisms of malignant mesothelioma carcinogenesis and provide a rationale for the development of novel malignant mesothelioma therapies targeted to selective molecular targets.

## Materials and Methods

**Cell cultures.** We used two established malignant mesothelioma cell lines (MMB and MMP) derived from pleural effusions of malignant mesothelioma patients and three primary HMC cultures obtained from patients with congestive heart failure; cells were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS, Life Technologies, Rockville, MD) at 37°C in a 5% CO<sub>2</sub>-humidified atmosphere. Mesothelial origin was confirmed by immunocytochemistry using antibodies against cytokeratin, vimentin, calretinin, and carcinoembryonic antigen. A profile of cytokeratin, vimentin, calretinin positivity and CEA negativity was established as criteria of mesothelial origin, as previously described (12). We stably transfected HMC cells with Tag-encoding cDNA or SV40 full-length DNA, using the polycation compound "Superfect" (Qiagen, Valencia, CA). The defective Tag mutant, indicated in the text as "TagRb-", carries the E107 → K amino acid substitution impairing Tag binding to Rb protein.

**Asbestos fibers and glass beads.** Size distribution of UICC asbestos samples were characterized according to Kohyama et al. (18). Chrysotile: length, 92% with  $L \leq 10 \mu\text{m}$ ; width, 90% with  $D \leq 0.32 \mu\text{m}$ ; 50% with  $D \leq 2.3 \mu\text{m}$ ; and 50% with  $D \leq 0.17 \mu\text{m}$ . Amosite: length, 90% with  $L \leq 20 \mu\text{m}$ ; width, 90% with  $D \leq 0.75 \mu\text{m}$ ; 50% with  $L \leq 4.0 \mu\text{m}$ ; 50% with  $D \leq 0.33 \mu\text{m}$ . We suspended amosite, crocidolite and glass fibers in PBS at 2.0 mg/mL before triturating them eight times through a 22-gauge needle and autoclaving.

**Cytotoxicity.** Cells were cultured for 24 hours in medium containing 2% FBS, supplemented with 10  $\mu\text{g}/\text{cm}^2$  amosite fibers, 10  $\mu\text{mol}/\text{L}$  H<sub>2</sub>O<sub>2</sub> or 100  $\mu\text{mol}/\text{L}$  etoposide (VP-16), in the presence or absence of purified HGF (50 ng/mL; R&D Systems, Inc., Minneapolis, MN). We also cultured SV40-HMC cells for 60 days, after two cycles of treatment, 72 hours each, with low concentrations (2.5  $\mu\text{g}/\text{cm}^2$ ) of amosite fibers ("long-term" exposure). Cytotoxicity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (19).

**Note:** P. Cacciotti and D. Barbone contributed equally to this work.

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**Apoptosis assay.** We exposed subconfluent cells to HAM's medium supplemented with 2% FBS and containing different apoptotic stimuli (10  $\mu\text{g}/\text{cm}^2$  amosite, 10  $\mu\text{mol}/\text{L}$   $\text{H}_2\text{O}_2$ , or 100  $\mu\text{mol}/\text{L}$  VP-16), in the presence or absence of HGF (50 ng/mL) or anti-HGF blocking antibodies (10  $\mu\text{g}/\text{mL}$ ; monoclonal antibody 294, R&D Systems). After incubation, cell pellets were stained with 2  $\mu\text{L}$  of propidium iodide (PI, final concentration 1.0  $\mu\text{g}/\text{mL}$ , Sigma, St. Louis, MO) and 2  $\mu\text{L}$  of FITC labeled-Annexin V (Alexis, Lausen, Switzerland), and 5,000 events per sample were analyzed by flow cytometry. Cells with Annexin V-positive staining only were scored as apoptotic. We evaluated caspase activity and CD95 expression by staining cells with CaspACE FITC-VAD-FMK *in situ* marker (Promega, Madison, WI) and CD95-FITC antibodies (Chemicon, Temecula, CA), respectively.

**Biochemical assays.** Immunoblotting was done as previously described (12), by loading 50  $\mu\text{g}$  of cell lysates in reducing conditions. After separation on SDS-PAGE and transfer to nitrocellulose filters (Hybond, Amersham, Buckinghamshire, United Kingdom), we probed filters with phospho-Akt (Ser<sup>473</sup>, Cell Signaling Technology, Beverly, MA), phospho-Erk1/2 (Sigma), phosphotyrosine (UBI, Lake Placid, NY), Bcl-2 and Bax antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and detected signals by the enhanced chemiluminescence system (Amersham). We used inhibitors (both from Sigma) of PI3K (Wortmannin) and Erk1/2 (PD98059) at a final concentration of 50 nmol/L and 30  $\mu\text{mol}/\text{L}$ , respectively, unless otherwise indicated.

**Cell cycle analysis.** After washing in PBS and fixing in ethanol, cells were stained for 30 minutes at room temperature with 50  $\mu\text{g}/\text{mL}$  PI in 0.1 mol/L PBS (pH 7.2) containing 0.5 mg/mL RNase. We analyzed 10,000 events per sample by flow cytometry.

**Immunohistochemistry.** A malignant mesothelioma tissue microarray (TMA) was provided by the Tissue Procurement Facility at Fox Chase Cancer Center (Philadelphia, PA). The TMA contained tissue cores from two different regions of each of 19 tumor specimens from primary malignant mesothelioma patients. Cores from normal tissue (lung, colon, and kidney) were used as negative controls. Slides containing formalin-fixed, paraffin-embedded samples were deparaffinized, hydrated in water, and subjected to antigen retrieval in 10 malignant mesothelioma citrate buffer (pH 6.0). Preparations were incubated in 3%  $\text{H}_2\text{O}_2$  for 20 minutes, washed with  $\text{H}_2\text{O}$  or PBS, and blocked with 10% serum for 30 minutes. Primary antibodies for phospho-Akt Ser<sup>473</sup>, SV40 Tag (pAB101), and Met C-28 (each from Santa Cruz Biotechnology) were detected with biotinylated secondary antibodies. Tissue sections were stained with 3,3'-diaminobenzidine chromogen and counterstained with hematoxylin. Surrounding nonneoplastic stroma served as an internal negative control for each slide.

**Statistics.** Data are expressed as mean percentage  $\pm$  SD of three independent experiments. We used the two-tailed Mann-Whitney test to evaluate statistical differences.

## Results

**Asbestos fibers induce time- and dose-dependent cytotoxicity in human mesothelial cells.** After exposure of HMC to asbestos, we observed that amosite and crocidolite were similarly toxic and induced significantly higher cytotoxicity than control glass fibers. Cytotoxicity was observed as early as 4 hours after initial exposure and progressively increased until 24 hours (Fig. 1A). We exposed HMC to increasing concentrations of amosite or crocidolite fibers for 24 hours and cytotoxicity was evident with both fiber types, starting at the lowest concentration (1  $\mu\text{g}/\text{cm}^2$ ) and progressively increasing up to a plateau of about 50% at 10  $\mu\text{g}/\text{cm}^2$  (Fig. 1B). These results show that amosite and crocidolite fibers are comparably toxic in HMC in a time- and dose-dependent manner.

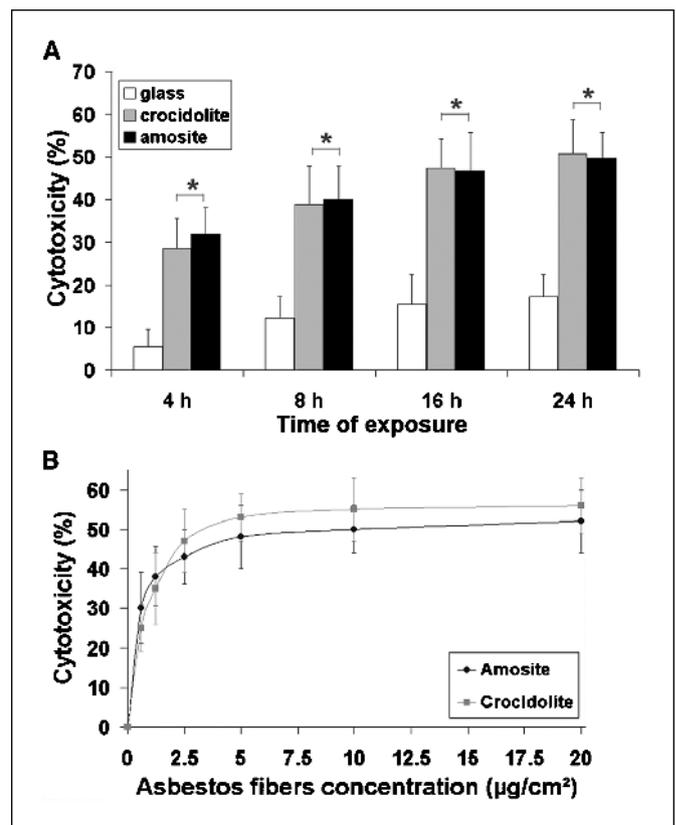
**Tag expression protects human mesothelial cells and malignant mesothelioma cells from asbestos-induced cytotoxicity.** We hypothesized that a growth factor autocrine loop induced by SV40 could positively affect HMC survival after exposure to toxic

agents. Based on our previous findings (12), we exploited the HGF autocrine loop as a paradigmatic survival pathway for HMC expressing SV40 Tag. Therefore, we used malignant mesothelioma cells characterized before and after culture establishment for the presence of SV40 sequences and expression of HGF.

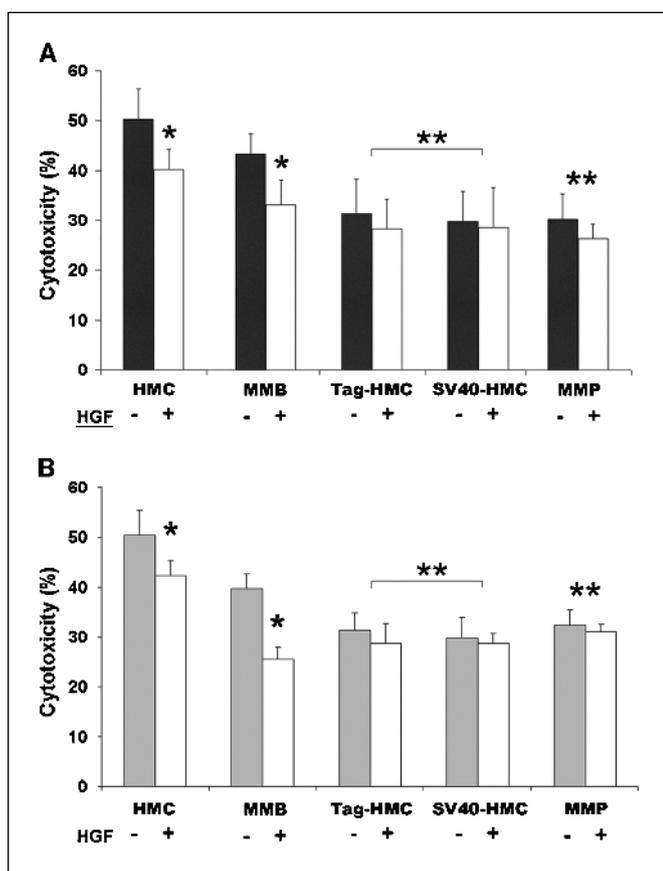
Cytotoxicity in response to amosite or crocidolite fibers was measured in HMC and malignant mesothelioma cells in the presence or absence of HGF stimulation. HMC and SV40-negative malignant mesothelioma cells (MMB), when stimulated with HGF, were significantly ( $P < 0.01$ ) less sensitive to asbestos-induced cytotoxicity than unstimulated cells. In contrast, HMC expressing recombinant Tag (Tag-HMC or SV40-HMC) and SV40-positive malignant mesothelioma cells (MMP) were intrinsically more resistant to asbestos induced cytotoxicity, irrespective of the presence of exogenous HGF (Fig. 2).

From these data, we conclude that the constitutive release of survival growth factors (e.g., HGF) by Tag expressing cells may establish a marked resistance to both toxic fibers. As cytotoxicity induced by amosite and crocidolite is consistently antagonized by HGF, hereinafter we tested amosite fibers in all experiments.

**Cell survival is mediated by phosphatidylinositol-3 kinase/Akt signaling.** We next stimulated HMC and SV40-negative MMB cells with HGF and VEGF, which have been reported to be induced by SV40 in malignant mesothelioma cells (12, 15, 20). These growth factors elicited Akt and Erk1/2 activities in HMC and SV40-negative MMB cells (Fig. 3A-B), and similar results (data not shown)



**Figure 1.** Asbestos-induced cytotoxicity is time and dose dependent in HMC. A, time course analysis of the cytotoxic effect on HMC exposed to glass, crocidolite, or amosite fibers in a range of 4 to 24 hours. B, dose-response cytotoxicity curve of HMC exposed to amosite or crocidolite. Points, means of three experiments; bars,  $\pm$ SD. \*,  $P < 0.001$ , significantly different from treatment with glass fibers.



**Figure 2.** Tag protects from asbestos-induced cytotoxicity. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on HMC, transfected Tag-HMC, SV40-HMC, and malignant mesothelioma cells exposed to amosite (A) or crocidolite (B) fibers, in the presence or absence of purified recombinant human HGF. Columns, means of three separate experiments; bars,  $\pm$ SD. \*,  $P < 0.01$ , significantly different compared with fiber exposure in absence of HGF. \*\*,  $P < 0.01$ , significantly different compared with HMC and MMB, respectively.

were obtained with IGF-I, also associated with SV40 infection (14). Interestingly, ligand-independent, elevated Akt activity was detected in unstimulated MMB cells transfected with cDNA encoding SV40 early region sequences (data not shown). Consistently, significant autonomous Akt and Erk1/2 activities were detected in Tag-HMC and SV40-positive malignant mesothelioma cells (MMP), which were markedly and selectively inhibited by treatment with the inhibitory drugs Wortmannin or PD98059, respectively (Fig. 3C-D). Under our conditions, asbestos activated Akt only transiently, and when cells were exposed to asbestos fibers in the presence of the PI3K inhibitor Wortmannin, cytotoxicity significantly ( $P < 0.001$ ) increased in both Tag-expressing and HGF-treated cells but not in HMC and SV40-negative MMB cells (Fig. 3E). In contrast, treatment with PD98059 did not alter cytotoxicity of HMC or malignant mesothelioma cells tested, although immunofluorescence analysis revealed that Erk1/2 translocation to the nucleus was abrogated (data not shown).

Altogether, these results indicate that PI3K/Akt but not mitogen-activated protein kinase (MAPK)/Erk1/2 signaling protects cells of mesothelial origin from asbestos-induced cytotoxicity. We conclude that Akt is a common critical pathway, whose activation mediates SV40-dependent survival in mesothelial cells.

### Human malignant mesothelioma tumors exhibit concordance between expression of Tag and phosphorylation of Akt.

Immunohistochemical analysis of a human malignant mesothelioma tumor TMA provides supporting evidence that SV40 Tag may contribute to the activation of Akt. Tag expression was evident in 11 (58%) of 19 malignant mesothelioma tumors examined, as punctuate nuclear staining, whereas Tag was not detected in normal tissues. Positive staining for phosphorylated (active) Akt was observed in 10 of 11 tumors exhibiting Tag expression. A representative malignant mesothelioma tumor with Tag and phospho-Akt positivity is depicted in Fig. 3F. Statistical assessment of the immunohistochemical staining, summarized in Fig. 3G, revealed that Tag positivity is associated with phospho-Akt positivity ( $P = 0.0408$ ).

**Phosphatidylinositol-3 kinase/Akt protects human mesothelial cells from apoptosis.** Asbestos fibers are known to induce apoptosis in human and rabbit mesothelial cells via oxidative stress (8). We examined cell survival of HMC, Tag-HMC, and TagRB-HMC in response to amosite or VP-16. TagRB-HMC cells express Tag defective for binding to Rb protein, which is required to induce HGF expression (12). By flow cytometry analysis, we found that the two proapoptotic agents increased the sub- $G_1$  (apoptotic) fraction of HMC, Tag-HMC, and TagRB-HMC. Amosite and VP-16 induced higher apoptosis rate in TagRb-HMC than in Tag-HMC, consistent with the impaired Rb binding of the Tag mutant. Moreover, HGF-blocking antibodies almost completely abrogated the protective effect in Tag-HMC but not in TagRb-HMC (Fig. 4A). Interestingly, the level of amosite-induced apoptosis observed in Tag-HMC and TagRB-HMC was significantly lower than that of HMC. This strongly suggests that factors in addition to HGF are involved, consistent with the higher level of Akt activity seen in TagRB-HMC than in HMC (Fig. 4A, top right inset).

To investigate the effectors of Akt-mediated survival, we examined apoptotic markers for caspase activity and CD95 FasL-receptor expression (21). Upon treatment of cells with VP-16, caspase activity increased markedly in HMC and MMB cells, whereas a lower caspase induction was observed in Tag-HMC and MMP cells. When cells were treated with both VP-16 and HGF, caspase activity was inhibited in HMC and MMB cells ( $P < 0.01$ ). Moreover, consistent with Akt activation, Wortmannin almost completely restored caspase activity in HGF-treated HMC and MMB cells as well as in Tag-HMC and MMP cells (Fig. 4B), although caspase activity was significantly lower in Tag-positive malignant mesothelioma cells than in Tag-negative malignant mesothelioma cells, reinforcing the hypothesis of multiple Tag-dependent survival effectors.

We also examined CD95 expression using flow cytometry. Upon VP-16 treatment, CD95 expression was high in HMC and MMB cells and low in Tag-HMC and MMP cells. Akt activation by HGF strongly inhibited CD95 expression in Tag-negative cells, but simultaneous addition of Wortmannin and HGF restored CD95 expression. Moreover, Wortmannin treatment led to a significant ( $P < 0.01$ ) increase in CD95 expression in Tag-HMC and in MMP cells (Fig. 4B). Bcl-2 and Bax expression did not undergo detectable changes in the same experiments (data not shown).

We conclude that Akt activation inhibits apoptosis in Tag-expressing mesothelial cells exposed to toxic agents, including amosite fibers, and down-regulates caspase activity as well as CD95 expression. These effects are strictly HGF-dependent in Tag-negative cells, whereas in both Tag-positive HMC and malignant mesothelioma cells other growth factors and/or other

Tag-dependent survival effectors are likely to be involved (22). Despite these differences, Akt inhibition consistently plays a pivotal role in restoring higher susceptibility to cell death after exposure to toxic agents.

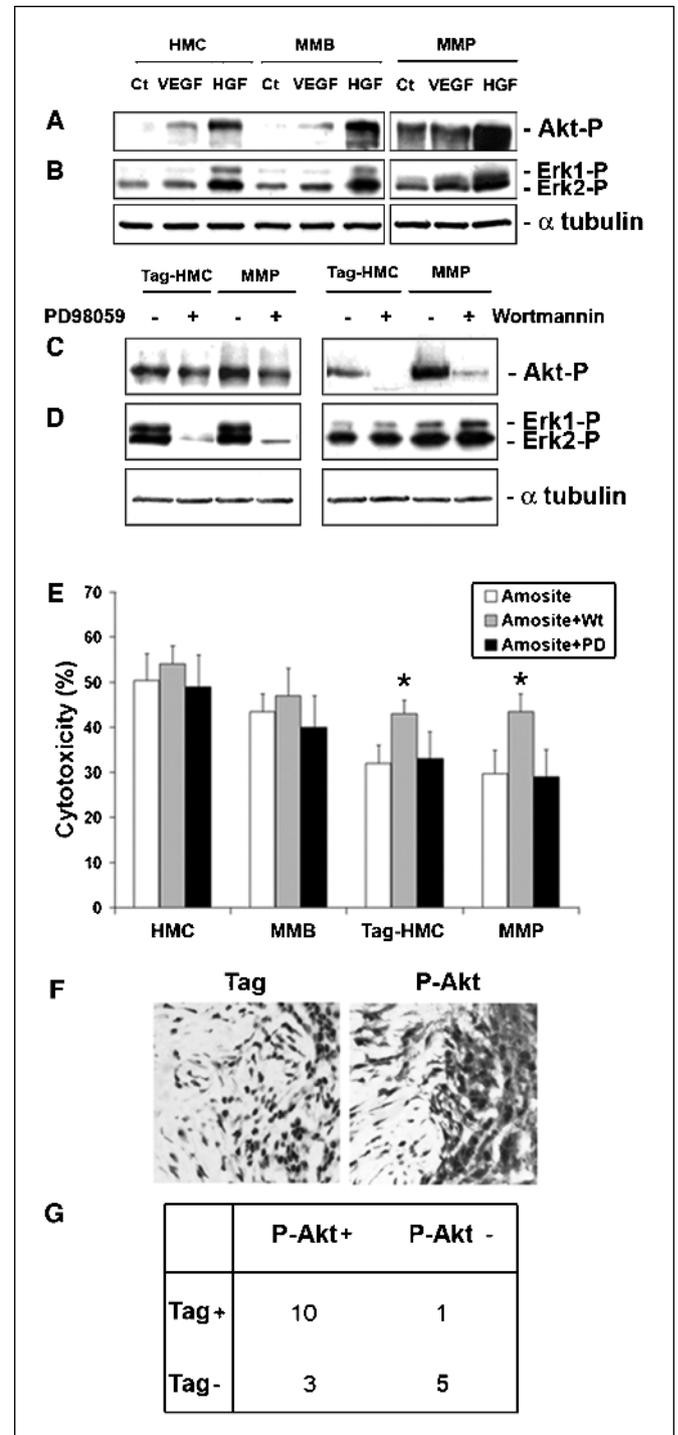
**Long-term exposure of SV40/human mesothelial cell to amosite fibers promotes cell transformation.** It has been shown that prolonged exposure of SV40-positive HMC to amosite may induce transformation (10). To verify whether the expression of Tag or the entire SV40 genome could influence mesothelial cell transformation, we treated SV40- or Tag-transfected HMC long term (see Materials and Methods). Thirty days after exposure, cells acquired striking morphologic changes, becoming larger and more vacuolated (Fig. 5A, middle) than the same cells were 7 days after treatment (Fig. 5A, left). After 60 days, SV40-HMC, but not Tag-HMC, underwent loss of cell contact inhibition and generated several *foci* (Fig. 5A, right). Cell cycle analysis after 30 days of treatment revealed that most surviving cells were blocked in G<sub>2</sub> phase. In contrast, cells obtained from *foci* escaped serum-dependency for growth and exhibited a strong increase in the S-phase entry (Fig. 5B), with activation of Akt (Fig. 5B, right inset). Moreover, these cells were progressively less sensitive to amosite during the time span of the long-term experiment (Fig. 5C). Interestingly, HGF protection decreased after 30 days and was lost after 60 days in culture, as well as in *foci* cells. Control HMC and Tag-HMC exposed to amosite did not survive long enough to complete the entire time span of the experiment. Noteworthy, inhibition of Akt activity by treatment with 50 nmol/L Wortmannin in cells derived from *foci* significantly restored the cytotoxic response (Fig. 5D). All experiments were done on cells derived from several independent *foci*. Cells cultured over seven passages maintained the *in vitro* transformed phenotype described above. Based on these findings, we conclude that prolonged exposure to low doses of amosite fibers induces cell transformation of SV40-HMC via Akt activation.

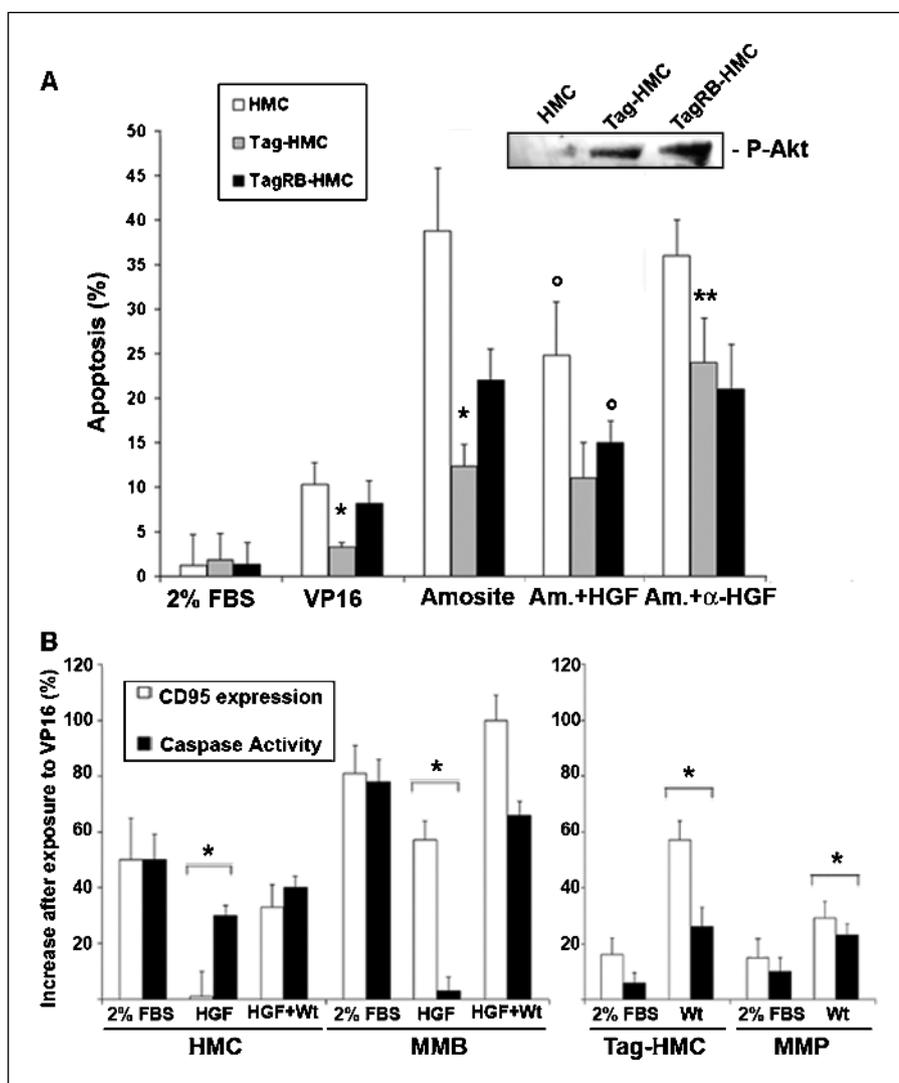
**Discussion**

In the present work, we show that the PI3K/Akt signaling pathway is responsible for resistance to cell death in HMC and malignant mesothelioma cells after amosite exposure. Upon exposure to toxic agents, we show that the PI3K/Akt pathway is activated in response to growth factors in the presence of Tag and confers progressive resistance to apoptosis as well as abrogation of CD95/Fas up-regulation. On the other hand, inhibition of Erk1/2 activity had no effect on HMC cytotoxicity, albeit translocation of MAPK to the nucleus was abrogated (data not shown).

**Figure 3.** Tag-induced protection against apoptosis is dependent on PI3K/Akt signaling. Immunoblotting on total cell lysates of Tag-negative (HMC and MMB) and Tag-positive MMP cells exposed to HGF or VEGF (A-B) and of Tag-positive cells treated with Erk1/2 inhibitor PD98059 or PI3K inhibitor Wortmannin (C-D), done with phospho-Akt (Ser<sup>473</sup>) antibodies (A-C) and phospho-Erk1/2 antibodies (B-D).  $\alpha$ -Tubulin immunoblotting were used as loading control. E, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on cells exposed to amosite fibers in the presence or absence of Wortmannin or PD98059. Columns, means of three experiments; bars,  $\pm$ SD. \*,  $P < 0.001$ , significantly different compared with fiber exposure without inhibitors. F, immunohistochemical staining of tumor cells from a representative human malignant mesothelioma with antibodies against SV40 Tag and phosphorylated Akt. Note stroma (left) and tumor (right) in each photograph, with strong positivity for Tag and phospho-Akt in tumor tissue. Magnification, 400 $\times$ . G, tabulation of overall immunohistochemical staining of a malignant mesothelioma tissue microarray with antibodies against Tag and phospho-Akt.

Our findings also support a role for HGF as an of SV40-dependent survival factor that cooperates with amosite fibers in malignant mesothelioma carcinogenesis. Moreover, addition of exogenous HGF exerts a protective effect on SV40-negative HMC exposed to toxic agents. The markedly higher rate of apoptosis occurring in TagRB-HMC than in Tag-HMC, when treated with VP-16 or amosite, provides further evidence of the role played by HGF, because the TagRB mutant lacks a functional Tag binding site for Rb, responsible for HGF release (12).





**Figure 4.** CD95 expression and caspase activity are down-regulated in Tag-positive cells. **A**, flow cytometry analysis of Annexin V-stained cells in response to control medium, VP-16, amosite fibers, recombinant HGF, or HGF-blocking antibodies. *Columns*, percentage of apoptotic cells (Annexin V positive;  $n = 3$ ); *bars*,  $\pm$ SD. \*,  $P < 0.01$ , significantly different compared with HMC. <sup>o</sup>,  $P < 0.01$ , significantly different compared with cells not exposed to HGF. \*\*,  $P < 0.01$ , significantly different compared with cells not exposed to HGF-blocking antibodies. **B**, flow cytometry analysis of CD95 expression and caspase activity. *Columns*, means increases of fluorescent cells ( $n = 3$ ) after exposure to VP-16; *bars*,  $\pm$ SD. Cells were grown in control medium (2% FBS) and in the presence of HGF, Wortmannin, or HGF + Wortmannin. \*,  $P < 0.01$ , significantly different compared with control medium.

Other evidence clearly shows that additional Tag-dependent mechanisms, other than HGF, must cooperate with amosite fibers to determine HMC transformation. Indeed, the low level of basal apoptosis in TagRB-HMC compared with HMC and the capability of other SV40-dependent growth factors (i.e., VEGF and IGF-1) to induce Akt activity address to this conclusion. Moreover, Tag has been shown to possess an intrinsic antiapoptotic capability (22).

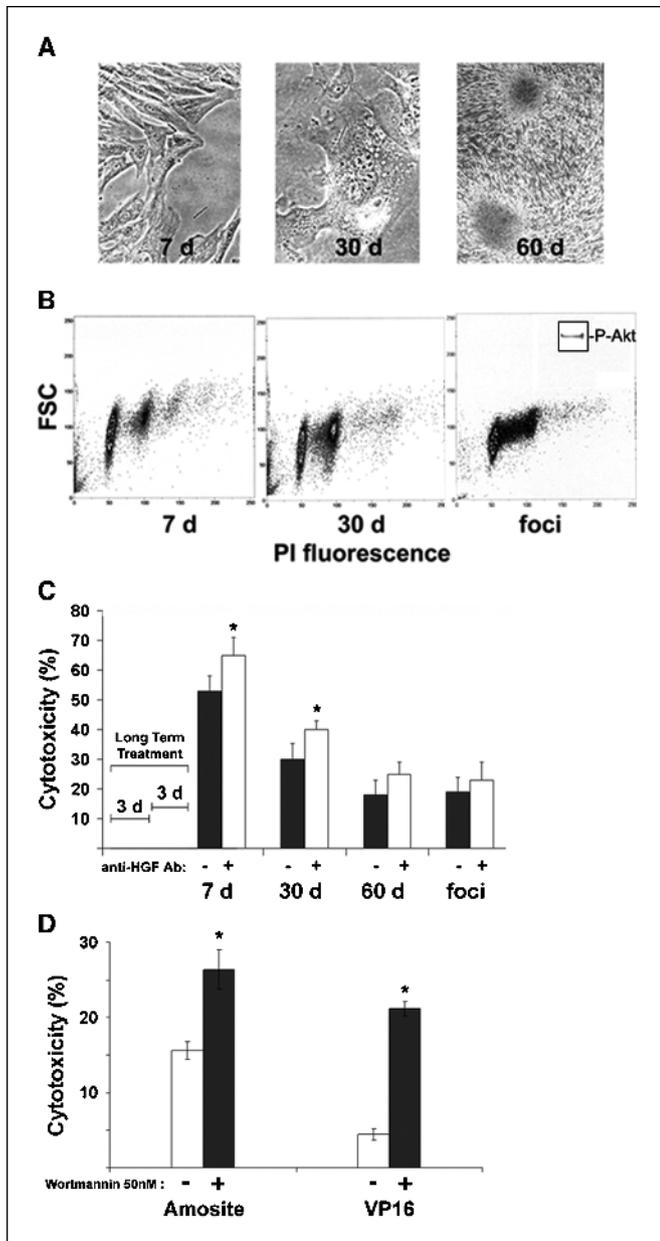
The protective role of HGF shown here is likely to play a crucial role in the *in vitro* transformation of SV40-negative HMC. Presumably, because no Tag-related autocrine loops are present in SV40-negative mesothelial cells, the effects elicited by HGF are more prominent. Our previous work had suggested a paracrine mechanism by which HGF can affect *in vivo* SV40-negative HMC (12). HGF is also relevant in the early stages of amosite-induced transformation of SV40-positive HMC. Transformed HMC undergoing DNA damage may enter into the S phase due to the loss of normal checkpoint control. However, during the transformation process, inhibition with HGF antibodies neither restored sensitivity to amosite cytotoxicity nor affected Akt activity. Altogether, these results suggest that transformed HMC become HGF-independent, and other unidentified mechanisms

contribute to the final steps of malignant mesothelioma carcinogenesis. Conversely, Akt activity seems consistently required to promote survival to amosite fibers, from the very early steps of *in vitro* transformation.

Previous work has shown that expression of the SV40 genome, or of Tag-encoding sequences, increases apoptosis resistance in various cell types (17, 23–25). Expression of recombinant Tag in human malignant mesothelioma cells exerts an antiapoptotic effect, which is reverted by Tag antisense RNA (26). In contrast, Tag-dependent apoptosis was observed after exposure of transfected rat mesothelial cells to crocidolite fibers (9). These discrepancies may be attributed to the different origins of the mesothelial cells and to different levels of Tag protein expression.

Several reports support the antiapoptotic effect of HGF described here (27), and our results confirm the link between the inhibition of caspases and HGF stimulation. Moreover, our findings provide *in vivo* evidence supporting HGF/Akt signaling in association with SV40 Tag expression in primary malignant mesotheliomas. A correlation of SV40, Akt activity, and CD95/Fas receptor down-regulation has already been reported (24, 25). Our data show that Akt activity inhibits CD95 overexpression subsequent to DNA damage by the chemotherapeutic agent VP-16,

providing a mechanistic link with the observed antiapoptotic effect. In these studies, Bcl-2 and Bax are unaffected by Akt activation, suggesting a minor role of Bcl-2 in regulating apoptosis in malignant mesothelioma cells (28, 29).



**Figure 5.** Amosite, SV40, and PI3K/Akt cooperate in mesothelial cell transformation. *A*, cell morphology of SV40-HMC at 7 days (7 d), 30 days (30 d), and 60 days (60 d) after long-term treatment with amosite. Original magnification,  $\times 320$ . *B*, cell cycle analysis at 7, 30 days, and in *foci* cells. *Inset*, representative immunoblot done with phospho-Akt (Ser<sup>473</sup>) on lysates obtained from *foci* cells. *C*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on SV40-HMC cells surviving after "long treatment" and exposed to amosite fibers, in the presence or not of HGF-blocking antibodies. *Columns*, means of three experiments; *bars*,  $\pm$ SD. \*,  $P < 0.05$ , significantly different compared with exposure without anti-HGF antibodies. *D*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on cells derived from *foci*, exposed to amosite fibers or treated with VP-16, in the presence or absence of Wortmannin. *Columns*, means of three experiments; *bars*,  $\pm$ SD. \*,  $P < 0.05$ , significantly different compared with exposure without Wortmannin.

We show here also that a long exposure of SV40-HMC to amosite leads to a progressive increase in cytotoxicity resistance and to substantive cell transformation at least *in vitro*, although the validation of the transforming phenotype by xenotransplant experiments will be conclusive. These findings provide supportive evidence that both Tag and small T antigen are required to exert the complete array of SV40's biological effects, including HMC transformation, as previously reported (10–12, 26).

Finally, our findings provide further evidence that PI3K/Akt activation is HGF responsive exclusively over the early phases of the transformation process, fully transformed cells and SV40-positive malignant mesothelioma cells being nonresponsive to HGF. On the other hand, HGF maintains its ability to activate the PI3K/Akt pathway in SV40-negative HMC and malignant mesothelioma cells. Amplification or selective overexpression of specific PI3K isoforms, as recently described in lung and other tumor cells (16, 30, 31), as well as the contribution of other cytokines (as discussed above) cannot be ruled out in fully transformed HMC and in SV40-positive malignant mesothelioma cells to account for Akt activation.

Our immunohistochemical analysis of human malignant mesothelioma specimens offers *in vivo* evidence that links Akt activity and expression of Tag. The activation of Akt also is likely to be mediated by autocrine or paracrine signaling involving growth factors such as HGF, because three malignant mesothelioma tumors expressing Met but not Tag also exhibited phospho-Akt activity (data not shown). Our results indicate that transfecting SV40 sequences into SV40-negative cells malignant mesothelioma (MMB) leads to Akt activation and augmented resistance to toxic fibers, which are significantly reverted by Wortmannin treatment.

Data presented here and in previous work (12) allow us to propose a model of SV40/amosite cocarcinogenesis involving both paracrine and autocrine mechanisms that lead to progressive HMC transformation. As recently proposed for all human malignant mesothelioma tumors (32), the selection of an amosite resistant stem HMC population induced by SV40-dependent and paracrine mechanisms may favor the expansion of transformed cells with growth advantage and higher proliferation rate, leading to the development of malignant mesothelioma.

Furthermore, our data show that PI3K/Akt signaling represents a potentially important *in vivo* pharmacologic target in malignant mesothelioma. In many human tumor types, activation of the PI3K/Akt pathway is a common event (33), suggesting that disruption of this signaling pathway may sensitize malignant mesotheliomas to chemotherapy. Given the very poor response of malignant mesotheliomas to chemotherapy (34) and the identification of SV40 sequences in a subset of malignant mesotheliomas, PI3K/Akt activation may serve as a biomarker for specifically tailored therapeutic approaches for malignant mesothelioma. Such drugs might well either interfere with mechanisms essential for malignant mesothelioma cell survival/tumor progression and sensitize malignant mesothelioma cells to the cytotoxic effects of standard chemotherapies.

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