Human mesothelioma cells exhibit tumor cell–specific differences in phosphatidylinositol 3-kinase/AKT activity that predict the efficacy of Onconase

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Abstract
Malignant mesothelioma is an aggressive cancer with no known cure, which has become a therapeutic challenge. Onconase is one of few chemotherapeutic agents that have been studied in patients with malignant mesothelioma that has the advantage of low toxicity and limited side effects. Here, we evaluate the effect of Onconase on killing of malignant mesothelioma cells and how the phosphatidylinositol 3-kinase/AKT (PI3-K/AKT) survival pathway influences this effect. Our results show that Onconase induces apoptosis in malignant mesothelioma cell lines and that this effect is tumor cell specific. Malignant mesothelioma cell lines with the highest AKT activation, which correlated with the presence of the SV40 large and small T antigen (SV40 + +), were the most resistant to the drug. Finally, a cooperative effect was observed between small molecule inhibitors of PI3-K and Onconase in the killing of malignant mesothelioma cells. Our results suggest that kinase screening of individual malignant mesotheliomas for endogenous levels of activated PI3-K/AKT may be predictive of the efficacy of Onconase and possibly other chemotherapeutic agents. [Mol Cancer Ther 2005;4(5):835–42]

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Introduction
The processes involved in the initiation and development of malignant mesothelioma, a malignancy derived from pluripotent mesothelial cells with a variety of tumor phenotypes, including epithelial, sarcomatous, and mixed varieties (1), are under intense investigation (2). This unique tumor has been associated historically with occupational exposures to amphibole types of asbestos (1, 3). In the past few years, SV40, a DNA virus, has been linked to the etiology of mesothelioma. Multi-institutional studies show that ~50% of human mesotheliomas in the United States contain SV40 large T-antigen DNA sequences (4, 5). Although malignant mesothelioma is a relatively rare cancer, its incidence is increasing in several countries (6). More important, the prognosis of patients with mesothelioma is grim as most survive <1 year after initial diagnosis (1, 3). Thus, effective therapeutic strategies are desperately needed.

Current treatment modalities include surgery, chemotherapy, and radiation therapy (7) and several new investigational approaches are now being tested, including intrapleural IFNγ, photodynamic therapy, immunotherapy, and gene therapy. Whereas some believe that radical surgical resection is the only chance of cure or meaningful improvement in survival, the majority of patients presenting with malignant mesothelioma are not candidates for radical surgical resection due to unresectable tumors or medical illness (8). As alternatives or complements, many chemotherapeutic agents have been studied in patients with malignant mesothelioma, either as single or combined agents, but low response rates have been found in most studies with the highest response rates generally achieved using combined treatment regimens (8). No clear standard of care has emerged and in most cases palliative treatment is used as the primary means of therapy (9).

One novel agent for the treatment of malignant mesothelioma is Onconase, a RNase derived from eggs and early embryos of the leopard frog (Rana pipens) that has shown activity against a variety of human tumors in vitro and in vivo (10). Onconase has been tested in phase I and phase II human clinical trials for treatment of numerous solid tumors, including malignant mesothelioma (11). Phase III trials of Onconase are still ongoing. Onconase is a homologue of RNA A, which preferentially degrades tRNA (12). It has been found that Onconase inhibits cell growth and proliferation and induces apoptosis through protein-synthesis inhibition-dependent and inhibition-independent mechanisms (11). One advantage of Onconase as an anticancer drug is that proliferating cells are more susceptible to it than quiescent cells (13), which could be one reason why Onconase is more toxic to cancer cells than...
to noncancer cells (10). The potential of RNases as cancer chemotherapeutic agents are enormous due to their low toxicity in humans. However, more research is needed to understand the mechanisms of resistance of malignant mesothelioma to these drugs, increase their efficacy, and be able to rationally combine them with other chemotherapeutic drugs.

In this study, we investigated the involvement of the PI3-K/AKT survival pathway in malignant mesothelioma resistance to Onconase. The PI3-K pathway is activated in many cancers (14) and we have recently shown that it is also frequently activated in human mesotheliomas where it can be targeted to inhibit mesothelioma cell growth.6 Here, we show that malignant mesotheliomas have different degrees of response to killing by Onconase. Those malignant mesothelioma cell lines with the most elevated AKT kinase activity, which correlates in most cases with positive SV40 status (SV40+), were less susceptible to inhibition of cell growth and survival by Onconase. Our results suggest that screening of malignant mesothelioma for cell survival–related kinases, particularly PI3-K/AKT, may be necessary in determining effective treatment of malignant mesotheliomas with Onconase.

Materials and Methods

**Human Mesothelial and Mesothelioma Cell Lines**

Human pleural mesothelioma cell lines (n = 9) were isolated from patients at autopsy and were kindly provided by Drs. Michele Carbone (Loyola University, Chicago, IL; MPP, MHF, and MCAT), Luciano Mutti (Maugeri Foundation, Pavia, Italy; MB and MMO), and Harvey Pass (Wayne State University, Detroit, MI; MGAR, MGAT, MMIL, M17). The MB, MMO, MGAR, MGAT, MMIL, and M17 lines were negative for SV40 large and small T-antigen (SV40–), and MHF, MPP, and MCAT were SV40+. All cell lines were tested for the mRNA expression of large and small T/antigen by PCR before each experiment. Cells were retrieved from frozen stocks and propagated in DMEM/F12 medium (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum, hydrocortisone (100 ng/mL), insulin (2.5 µg/mL), transferrin (2.5 µg/mL), and selenium (2.5 ng/mL; Sigma, St. Louis, MO).

**Small Molecule Inhibitors and Chemicals**

Stock solutions of the PI3-K inhibitor LY294002 were diluted in DMSO and used at nontoxic and specific concentration (2.5 ng/mL; ref. 15). LY294002 was obtained from Calbiochem (La Jolla, CA). Wortmannin (Sigma), and DMSO and used at nontoxic and specific concentration (2.5 ng/mL; Sigma, St. Louis, MO).

Growth Curves

Cells (n = 2–3 plates/group/time point) were plated at ~2 × 10^5 cells per 60-mm-diameter plate in complete medium, allowed to attach for 24 hours, and then treated with inhibitors at the indicated time points. Cells were removed by trypsination and aliquots counted using a hemocytometer to determine total cell number.

**Western Blot Analyses**

Nearly confluent malignant mesothelioma cells were washed thrice with cold PBS, scraped from the plates, and collected by centrifugation at 14,000 rpm for 1 minute. The pellet was resuspended in lysis buffer [20 mmol/L Tris (pH 7.4), 1% Triton X-100, 10% glycerol, 137 mmol/L NaCl, 2 mmol/L EDTA, 25 mmol/L HEPES, 1 mmol/L Na3VO4, 2 mmol/L L-PI, 1 mmol/L phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, 1 mmol/L DTT, 10 mmol/L NaF, 1% aprotinin], incubated at 4°C for 15 minutes, and centrifuged at 14,000 rpm for 20 minutes. Protein concentrations were determined using a Bio-Rad assay (Bio-Rad, Hercules, CA). Thirty micrograms of protein in sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mmol/L DTT, 0.1% w/v bromophenol blue] was resolved by electrophoresis in 10% SDS-polyacrylamide gels and transferred to nitrocellulose using a semidy transfer apparatus (Ellard Instrumentation, Ltd., Seattle, WA). Blots were incubated in blocking buffer (TBS containing 5% nonfat dry milk plus 0.1% Tween 20; Sigma) for 1 hour, washed thrice for 5 minutes each in TBS/0.1% Tween 20, and incubated at 4°C overnight with antibodies specific to p-AKT or AKT, both at a 1:500 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Blots were then washed thrice with TBS/0.1% Tween 20 and incubated with a specific peroxidase-conjugated secondary antibody for 1 hour. After washing blots thrice in TBS/0.1% Tween 20, protein bands were visualized with the LumiGlo enhanced chemiluminescence detection system (Kirkgaard and Perry Laboratories, Gaithersburg, MD) and quantitated by densitometry (17). Blots were reprobed with an antibody to β-tubulin in a dilution 1:1,000 (Santa Cruz Biotechnology) to validate equal loading between lanes (18).

**Kinase Activity Assays**

Protein kinases were immunoprecipitated from whole cell lysates (300 µg) prepared from near-confluent malignant mesothelioma cells using an AKT antibody (2 µg; Santa Cruz Biotechnology), then washed and incubated for 20 minutes at 30°C in kinase buffer [20 mmol/L HEPES (pH 7.5), 2 mmol/L L-2-mercaptoethanol, 5 mmol/L MgCl2 containing 5 µCi [γ-32P]ATP and 5 µg protein kinase Ba substrate per reaction (Calbiochem). Incorporation of 32P into the substrate was visualized by autoradiography following SDS-PAGE and quantified using phosphoimaging (Bio-Rad Multi-Analysis program; ref. 18).


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Flow Cytometry

Nearly confluent cells were maintained in complete medium containing 0.5% fetal bovine serum overnight before addition of Onconase at 1 and 10 μg/mL medium. At 24, 48, and 72 hours, medium was removed and adherent cells harvested by trypsinization. Cells were resuspended at 10^6/mL in staining solution (50 μg/mL propidium iodide, 0.1% Triton X-100, and 32 μg/mL RNase A) in PBS and incubated for 30 minutes at 37°C before analysis of 10,000 cells/group/time point in triplicate. The distribution of cells, including cells with a hypodiploid DNA content indicative of apoptosis or necrosis, was determined using a Coulter Epics Elite flow cytometer and appropriate software as previously described (19).

Methods for Detection of DNA Strand Breaks and Apoptosis

Terminal deoxynucleotidyl transferase (TdT)–mediated nick end labeling (TUNEL) assays for detection of DNA strand breaks were done using a commercial kit following the manufacturer’s instructions (Promega Corporation, Madison, WI; ref. 20). Cells were plated on glass coverslips and grown to near confluence. After exposure to agents, slides were fixed in 10% buffered formalin for 25 minutes at room temperature, washed with fresh PBS several times, and cells permeabilized using 0.2% Triton X-100 solution in PBS. Slides then were incubated with 100 μL of equilibration buffer, a biotinylated nucleotide mix, and the TdT reaction mix at 37°C for 1 hour in a humidified chamber. The reaction was terminated with 2× SSC for 15 minutes. Endogenous peroxidases were blocked by immersing the slides in 0.3% H₂O₂ for 5 minutes. Slides then were incubated with streptavidin horseradish peroxidase complex for 30 minutes, stained with 3,3’-diaminobenzidine tetrahydrochloride, and counterstained with hematoxylin to detect apoptotic/necrotic nuclei, which were analyzed using transmission electron microscopy. Negative controls included cells incubated in enzyme alone and positive controls consisted of cells treated with DNase I (Promega). To determine the number of apoptotic cells, cells were stained with annexin V and propidium iodide in the dark and grown to near confluence. After exposure to agents, slides then were incubated with 100 μL of binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl₂], 5 μL of propidium iodide at a final concentration of 2.5 μg/mL (Sigma), and 2 μL of FITC labeled-annexin V (Alexis, Firenze, Italy). Cells with annexin V–positive staining were scored as apoptotic.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay for Cell Viability

Assays were done on 96-well microtiter plates after plating of 7.7 × 10³ cells/well. Malignant mesothelioma cell lines were then cultured for 24 hours in complete medium before changing to medium containing 0.5% fetal bovine serum. Cells were then treated with LY294002 or DMSO (solvent control) 1 hour before the addition of Onconase (10 μg/mL). Cell viability was assessed using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (R&D Systems, Minneapolis, MN), in which 0.5 mg/mL of MTT was added to the wells for 3 to 4 hours before dissolution in DMSO and its absorbance read at 570 and 650 nm. ΔAbsorbance from these two wavelengths are reported as the corrected viability. Fold changes were calculated with respect to the control.

Statistical Analyses

In all experiments, duplicate or triplicate determinations were conducted for each group (n = 2-3) per time point. Experiments were done in duplicate. Results were evaluated by one-way ANOVA using the Student-Newman-Keuls procedure for adjustment of multiple pairwise comparisons between treatment groups. Differences with \( P \leq 0.05 \) were considered statistically significant.

Results

Onconase Differentially Affects Mesothelioma Cell Growth

Figure 1 shows the effect of Onconase at 1 and 10 μg/mL on growth of 4 malignant mesothelioma cell lines (MB/MMO as SV40– cell lines and MPP/MCAT as SV40+ cell lines). The effect of Onconase was tumor-specific, with the SV40+ cell lines showing an increased resistance to the drug. These studies showed that Onconase at both 1 and 10 μg/mL caused significant (\( P \leq 0.05 \)) dose-related killing at 24 to 72 hours in MB cells (Fig. 1A) and at 48 and 72 hours in MMO cells (Fig. 1B). Onconase at these same concentrations did not significantly affect the growth of MPP and MCAT (SV40+) cell lines (Fig. 1C and D).

Onconase Causes Dose-Related Increases in Proportions of Hypodiploid (sub-G₀/G₁) Mesothelioma Cells Over Time That Is Reflected in Increased Cell Killing and TUNEL Positivity

To examine the effect of Onconase on cell growth, we determined the effects of the drug (1 and 10 μg/mL) on cell cycle kinetics of these four malignant mesothelioma cell lines over a 72-hour period using flow cytometry. In comparison to the more resistant MPP and MCAT SV40+ cell lines, the MB and MMO lines exhibited significant increases (\( P \leq 0.05 \)) in the fraction of the cell population in sub-G₀/G₁, which increased over time (Fig. 2A–D). These changes occurred with concomitant decreases in cells in G₀/G₁ (Fig. 2E–H).

To determine the mechanism of cell death by Onconase in malignant mesothelioma, control and Onconase-treated MB and MMO SV40– cell lines were examined using the TUNEL assay, which detects apoptotic and necrotic cell death. In comparison to sham control cells (Fig. 3A and C) or cells incubated without TUNEL enzyme (control in Fig. 3C), TUNEL-positive cells were observed in both mesothelioma cell lines exposed to Onconase (10 μg/mL for 48 hours; Fig. 3B and D) and in MB cells pretreated with DNase (+ control in Fig. 3F). Although TUNEL-positive cells often exhibited smaller nuclei indicative of apoptosis (Fig. 3B and D, arrows), transmission electron microscopy of adherent cells treated with Onconase also indicated lytic cell death (data not shown).
AKT Activity Is Increased in SV40+ Malignant Mesothelioma Cell Lines

To establish whether the AKT survival pathway was a factor in resistance to Onconase in SV40+ malignant mesothelioma cells, we studied the status of AKT activity in nine malignant mesothelioma cell lines. After a 24-hour period in low serum (0.5% fetal bovine serum) medium and addition of 10% fetal bovine serum for 4 hours, increased phosphorylated AKT were observed in the SV40+ cell lines, MCAT, MHF, and MPP, compared with the SV40/C0 cell lines (Fig. 4A). Using a kinase activity assay, we confirmed in four malignant mesothelioma lines that AKT activity was higher in SV40+ cell lines (Fig. 4B). These results suggested that the efficacy of Onconase on killing of malignant mesotheliomas may be dependent on the extent of endogenous PI3-K/AKT pathway activity.

Inhibition of PI3K/AKT Increases Cell Death Induced by Onconase

A wide range of extracellular signals can activate PI3K and trigger the phosphorylation of protein kinase B (AKT), providing increased cell survival. Based on results presented above, we hypothesized that AKT activation would protect malignant mesothelioma cells from killing by Onconase. Figure 5 shows the survival patterns, using the MTT assay, of MB (SV40−) and MPP (SV40+) cell lines over a 72-hour period when treated with LY294002 alone (20 μmol/L), Onconase alone (10 μg/mL), or both in combination. The MB (SV40−) cell line, with the lower basal AKT activity, had the higher response to Onconase and a lower response to LY294002 (Fig. 5A). The MPP (SV40+) cell line, with the higher basal AKT activity, had a lower response to Onconase and the higher response to LY299402 (Fig. 5B). Both cell lines exhibited decreased survival with coexposure to LY294002 (20 μmol/L) and Onconase (10 μg/mL) at 48 and 72 hours. To verify if these results reflected cooperative inhibition of apoptosis by these agents, the effects of wortmannin (50 nmol/L) and Onconase (1 and 10 mg/mL), alone and in combination, were evaluated for annexin V−positive cells by flow cytometry. Apoptosis was significantly higher (P ≤ 0.01) in the combined treatment groups (wortmannin + Onconase) at both concentrations of Onconase (Fig. 5C).

Discussion

Malignant mesothelioma is a major therapeutic challenge. Many chemotherapeutic agents have been studied in patients with malignant mesothelioma, either as single agents or as part of a combination chemotherapy regime, with the highest response rates generally achieved using multiagent regimes (8). Malignant mesothelioma clinical trials for Onconase, a drug with low toxicity and limited side effects (13), have shown that some patients benefit from it whereas others do not (11). The biological reasons underlying differences in tumor response are unclear and undoubtedly complex. However, the observation that endogenous AKT activity correlates with malignant mesothelioma resistance to Onconase provides a plausible explanation for the differences observed here. We are currently validating activation of AKT pathway in SV40+ and SV40− primary tumors using immunofluorescence approaches on tissue arrays and primary cell cultures.
In this study, we evaluated the effect of Onconase on killing of malignant mesotheliomas in cell culture, and how the PI3-K/AKT survival pathway may influence these responses. Onconase affected cell growth and survival of malignant mesotheliomas in a differential manner, with those cell lines with higher AKT activity being the most resistant to the drug. One of the intracellular signaling pathways frequently activated in cancer is the PI3-K pathway (14). Downstream targets of the PI3-K pathway include serine/threonine kinases, such as the three isoforms of AKT (or PKB). In recent years, increasing evidence has implicated the PI3-K/AKT pathway in the regulation of cell size, proliferation, and survival, as well as tumor metastasis, angiogenesis, and

Figure 2. Onconase causes dose-related increases in proportions of hypodiploid (sub-G0/G1) and decreases in G0/G1 in mesothelioma cells over time. Flow cytometric analyses (A–H) showing cycle distribution in sub-G0/G1 (A, C, E, G) and G0/G1 (B, D, F, H) after exposure to Onconase (1 and 10 g/mL) in human mesothelioma cell lines SV40−: MB (A, B) and MMO (C, D) and SV40 +: MPP (E, F) and MCAT (G, H). *P < 0.05 compared with sham control cells at the same time.
invasiveness (14, 21–23). Recently, we have shown that the PI3-K/AKT pathway is frequently activated in human mesotheliomas and can be targeted to inhibit mesothelioma cell growth.5 Here, we present evidence that human mesothelioma cell lines with higher constitutive AKT activity are more resistant to Onconase. High AKT activity of mesothelioma cells correlated with SV40+ status. In recent years, it has been postulated that SV40 act as a cocarcinogen with asbestos in the pathology of mesothelioma (2, 24–27), although the pathogenic mechanisms are unclear. We suggest that SV40 may cooperate with asbestos in the development of malignant mesothelioma by increasing the activity of the PI3-K/AKT survival pathway. The PI3-K/AKT pathway can be activated by asbestos or SV40 Tag, which uses the PI3-K pathway to increase cell survival and/or inhibit apoptosis (28). To further support our results, it was shown recently in human mesotheliomas that the hepatocyte growth factor receptor (c-Met) is activated by SV40 (29). Previously, we also showed that hepatocyte growth factor can activate the PI3-K/AKT pathway in malignant mesotheliomas.5 These results suggest that SV40 might activate the PI3-K pathway through c-Met or other receptor-mediated mechanisms.

Our studies on the cooperative effects of cell killing using PI3-K inhibitors and Onconase suggest that

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**Figure 3.** Onconase causes cell death that is reflected in TUNEL positivity. TUNEL staining of untreated MB (SV40−; A) and MMO (SV40−; C) mesothelioma cells and those exposed to Onconase (10 μg/mL; B and D) for 48 h. E, untreated MB cells incubated without TUNEL enzyme (− control); F, MB cells pretreated with DNase before TUNEL staining (+ control). Arrowheads in (B) and (D) show TUNEL-positive cells.

**Figure 4.** Levels of AKT activity differ in human mesotheliomas. A, Western blot showing p-AKT/AKT expression. B, AKT kinase activity assay.
knowledge of the underlying signaling pathways involved in the survival of individual tumors might increase the efficacy of cell killing by chemotherapeutics. We surmise that initial screening of malignant mesotheliomas for activation of kinases important in tumor cell survival may be exploited to inhibit growth and development of malignant mesotheliomas and possibly other tumors. An understanding of the signaling pathways critical to malignant mesothelioma development and progression is critical for the design of more rational treatment options for patients.

References
Human mesothelioma cells exhibit tumor cell–specific differences in phosphatidylinositol 3-kinase/AKT activity that predict the efficacy of Onconase
