Antitumor activity of MEK and PI3K inhibitors against malignant pleural mesothelioma cells in vitro and in vivo

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Abstract. Malignant pleural mesothelioma (MPM) is an aggressive malignancy for which there is no approved targeted therapy. We examined the therapeutic efficacy of the mitogen-activated protein kinase kinase (MEK) and phosphatidylinositol 3-kinase (PI3K) inhibitors against human MPM cell lines both in vitro and orthotopically inoculated into severe combined immunodeficient (SCID) mice. In addition, the molecular mechanisms of these agents were confirmed in vitro and in vivo. The MEK or the PI3K inhibitor suppressed MPM cell growth in vitro in a dose-dependent manner via induction of G1 cell cycle arrest and apoptosis. In addition, combined use of the MEK and PI3K inhibitors showed an additive or synergistic inhibitory effect on MPM cell growth compared to treatment with either individual drug. Treatment with MEK or PI3K inhibitor suppressed the production of thoracic tumors and pleural effusion and prolonged the survival time of EHMES-10 cell-bearing SCID mice. The combination therapy more effectively prolonged the survival time compared to treatment with either individual drug. Immunohistochemical and western blot analysis of thoracic tumors suggested that these agents induced cell cycle arrest, apoptosis and inhibition of tumor angiogenesis. Our results suggest that a combination of MEK and PI3K inhibitors is a promising therapeutic strategy for MPM.

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive neoplasm that arises from mesothelial cells. It was reported that asbestos, iron, and simian virus 40 were linked to the etiology of MPM (1-4). MPM was once considered a rare disease, but its incidence is increasing worldwide (5).

Current aggressive multimodality therapy for MPM (consisting of surgical resection, cytotoxic chemotherapy, and radiation) offers survival benefits for only a small subset of patients in early stages of the disease (6). Recently, the multi-targeted antifolate pemetrexed has been approved as the front-line agent in combination with cisplatin for the treatment of MPM (7). However, most of the patients relapsed within a year after starting treatments. Therefore, new and more effective therapies are necessary to improve the prognosis of this disease.

The mitogen-activated protein (MAP) kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway and the phosphatidylinositol 3-kinase (PI3K)-Akt pathway play critical roles in the regulation of cell proliferation, growth, differentiation and survival (8-10). These pathways are activated in many types of solid tumor models, including MPM (8,9,11-13). It was also reported that inhibition of these pathways affected the proliferation of MPM cell lines in vitro (14,15). However, no report has demonstrated growth-inhibitory effects of these agents on MPM cells in vivo.

In the present study, we examined whether the MEK or the PI3K inhibitor affected the growth of MPM cells in vitro and in vivo. Furthermore, we evaluated the possibility that combined use of the MEK inhibitor and the PI3K inhibitor might enhance MPM treatment.

Materials and methods

Cell cultures. The human mesothelioma cell line EHMES-10 was established from the pleural effusion of a patient with MPM in our institution (16,17). MSTO211H was purchased from the American Type Culture Collection (Manassas, VA, USA). These cell lines were cultured in RPMI-1640 medium (Nikkense Bio Medical Laboratories, Kyoto, Japan) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), penicillin (100 U/ml) and streptomycin (50 μg/ml) in a 37°C humidified incubator with 5% carbon dioxide.

Reagents and inhibitors. MEK inhibitor U0126 and PI3K inhibitor LY294002 were purchased from LC Laboratories.
(Woburn, MA, USA). For in vitro experiments, these agents were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich Co., St. Louis, MO, USA) and were added to cells in medium with a final DMSO concentration of 1.0%. For in vivo studies, these agents were prepared as a suspension in a vehicle consisting of 40% DMSO in phosphate-buffered saline (PBS) (Wako Pure Chemical Industries, Osaka, Japan). Rabbit polyclonal antibodies against ERK1/2, phospho-ERK1/2, Akt, phospho-Akt, p27kip1, cyclin E, cyclin D1, p70S6K, phospho-p70S6K, S6, phospho-
S6, p90 ribosomal S6 kinase (p90RSK), phospho-p90RSK, glycogen synthase kinase-3β (GSK3β), phospho-GSK3β, Bad, phospho-Bad, poly(ADP-ribose) polymerase (PARP), procaspase 3, hypoxia-inducible factor 1α (HIF1α), and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibody against vascular endothelial growth factor (VEGF) was purchased from Millipore Co. (Tokyo, Japan). Mouse monoclonal antibody against CD31/platelet/endothelial cell adhesion molecule-1 was purchased from BD Pharmingen (Tokyo, Japan) for in vitro immunohistochemical study. Mouse monoclonal antibody against CD31 (PECAM-1) was purchased from Cell Signaling Technology for in vivo Western blot analysis. Horseradish peroxidase conjugated goat anti-rabbit IgG and horse anti-mouse IgG were purchased from Cell Signaling Technology.

**Cell proliferation assay.** The cell proliferation assay reagent WST-1 (4-[3-(4-lodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) (Roche Diagnostics GmbH, Mannheim, Germany) was used to assess the effect of U0126 or LY294002 on cell growth. MPM cells (1x10^4 cells/well) were plated in 96-well plates (Nunc, Roskilde, Denmark) and were exposed to various concentrations of test agents dissolved in DMSO. Controls received DMSO vehicle at a concentration equal to that of drug treated cells. After drug treatment for 72 h, 10 µl of WST-1 reagent were added to each well. Absorbance was measured at 450 nm with a reference wavelength of 690 nm by an E max precision microplate reader (Molecular Devices, Tokyo, Japan).

**Cell cycle analysis.** MPM cells, treated with or without test agents for 24 h, were trypsinized and collected, and the cell nuclei were stained using the CycleTest Plus DNA Reagent Kit (Becton-Dickinson, San Jose, CA, USA). Cells were subjected to FACScan analysis, and cell cycle profiles were determined using ModFitLT software (Becton-Dickinson, San Diego, CA, USA). This analysis was carried out independently three times.

**DNA fragmentation assay.** We examined DNA fragmentation to assess apoptosis in EHMES-10 or MSTO211H cells. Cells were treated with either U0126 or LY294002 or a combination of both for 24 h. DNA fragmentation was evaluated using the Cell Death Detection ELISA kit (Roche Molecular Biochemical, Indianapolis, IN, USA) as previously reported (18).

**Western blot analysis.** Cultured cells were treated with lysis buffer [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X, 50 mM NaF and 1 mM Na₃VO₄] containing proteinase inhibitor cocktail (Roche Diagnostics GmbH). Tumor tissue samples were homogenized in lysis buffer. Insoluble materials were removed by centrifugation at 4°C for 15 min 15,000 x g. Protein concentration was determined using a Bio Rad Protein Assay Kit (Bio Rad Laboratories, Hercules, CA, USA).

Proteins were separated on 7.5 to 15% polyacrylamide gels (Bio Rad Laboratories). After electrophoresis, the protein was transferred to a nitrocellulose membrane and detected by immunoblotting using SNAP i.d. Protein Detection System (Millipore Co.) as previously described (19). This analysis was carried out independently three times.

**Experimental animals.** Male severe combined immunodeficient (SCID) mice (six to eight weeks old) were obtained from Clea Japan (Osaka, Japan), fed autoclaved standard pellets and water, and maintained under specific pathogen-free conditions throughout this study. All of the protocols involving SCID mice were approved by the guidelines established by the Ehime University Committee on Animal Care and Use.

**Orthotopic implantation model.** Cultured EHMES-10 cells were harvested, washed twice and re-suspended in PBS. The SCID mice were inoculated in the thoracic cavity with the tumor cells (3x10^6 cells/mouse), as previously described (17,20). Seven days after inoculation, mice were randomized into eight groups (n=7 mice/group) to receive vehicle alone (DMSO + PBS), U0126 alone (20, 30 and 40 mg/kg), LY294002 alone (12.5, 25 and 50 mg/kg) and a combination of U0126 (30 mg/kg) and LY294002 (25 mg/kg). These agents were administrated intraperitoneally twice a week. Mice were sacrificed on day 30 after tumor cell inoculation. The tumor tissue was excised and weighed, and the volume of pleural effusion was measured. We also measured the body weights and serum levels of total protein (TP), blood urea nitrogen (BUN), creatinine (Cre), aspartate amino transferase (AST) and alanine aminotransferase (ALT), and evaluated the degree of dermatopathy as a measure of side effects.

**Immunohistochemistry.** Paraffin-embedded tissues were subjected to immunohistochemistry with anti-phospho-ERK1/2 monoclonal antibody, phospho-Akt monoclonal antibody, or anti-p27kip1 monoclonal antibody. For in situ apoptosis detection, we used terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL assay) with the In situ Apoptosis Detection Kit (Takara Biomedicals, Ohtsu, Japan). Frozen tissue sections were used for identification of endothelial cells using rat anti-mouse CD31/platelet/endothelial cell adhesion molecule-1 monoclonal antibody. Immunohistochemical procedures were performed using the Envision™ System (Dako, Glostrup, Denmark) method, as previously described (21). Phospho-ERK1/2- or phospho-Akt- or p27kip1-positive cells were visualized with Fuchsine substrate-chromogen (Dako). Antibodies against TUNEL assay or CD31 localization were detected using a peroxidase reaction with 3-diaminobenzidine (Dako).

**Statistical analysis.** In vitro study data are presented as means ± SD, and were analyzed using ANOVA followed by Dunnnett’s t-test. In vivo data were expressed as median values and ranges. The Mann-Whitney U test was used to compare groups. The Kaplan-Meier method was used to evaluate the survival analysis and comparisons were made using a log-rank test. Drug interactions were analyzed by the Chou and Talalay...
method using the CalcuSyn software program (version 2.0; Biosoft, Cambridge, UK). The combination index (CI) was simulated from each level of fractional affect. According to this method, a CI<0.3, 0.3-0.7, 0.7-0.9, 0.9-1.1, 1.1-1.45, 1.45-3.3 and >3.3 indicates highly synergistic, synergistic, moderate to slight synergistic, nearly additive, slight to moderate antagonistic, antagonistic and strong antagonistic, respectively. Differences between groups are considered statistically significant at P<0.05.

Results

Growth inhibition of MPM cells by U0126 and/or LY294002 treatment. The effects of U0126 or LY294002 at concentrations ranging from 20 to 200 µM on the proliferation of EHMES-10 or MSTO211H cells were determined with the WST-1 assay. Each agent inhibited MPM cell growth in a dose-dependent manner (Fig. 1A). The IC_{50} values for U0126 and LY294002 against EHMES-10 cells were 66.8 µM and 20.7 µM, respectively. Moreover, the IC_{50} values for U0126 and LY294002 against MSTO211H cells were 39.0 µM and 29.9 µM, respectively.

We evaluated the effect of combining treatments with U0126 and LY294002. The ratio of IC_{50} values for U0126 and LY294002 against EHMES-10 cells was approximately 3:1 while the ratio was 4:3 against MSTO211H cells. Therefore, the two MPM cell lines were exposed to varying concentrations of U0126 and LY294002 at fixed ratios of 3:1 or 4:3, as appropriate. Cell viability was then assessed by the WST-1 assay. The averaged CIs for EHMES-10 cells and MSTO211H cells were 1.017 and 0.54, which indicates a nearly additive effect and a synergistic effect, respectively (Fig. 1B).

Induced G1 cell cycle arrest of MPM cells after treatment with U0126 and/or LY294002. To investigate the mechanisms of growth inhibition of MPM cells by U0126 or LY294002 treatment, we performed cell cycle analysis of EHMES-10 cells or MSTO211H cells treated with 80 µM U0126 and/or 80 µM LY294002. Treatment with U0126 or LY294002 for 24 h significantly increased the G1-phase populations compared to control in both MPM cell lines (all, P<0.05) (Fig. 2A). In addition, U0126 alone and LY294002 alone significantly increased the percentage of MPM cells in the sub-G1 phase, indicative of cell apoptosis, compared to control (all, P<0.05). Combining treatment with U0126 with that of LY294002 led to a significant increase in the sub-G1 phase population in both cell lines compared to control or individual drugs (all, P<0.01).

We also analyzed the expression of cell cycle regulatory proteins after treatment with U0126 and/or LY294002 in both EHMES-10 cells and MSTO211H cells. Both agents increased p27kip1 expression and decreased cyclin E expression in both cell lines (Fig. 2B). A decrease of cyclin D1 expression was observed in treatment with either U0126 or LY294002 in EHMES-10 cells, and following treatment with U0126 in MSTO211H cells.
Induction of apoptosis by U0126 and/or LY294002 treatment. We assessed the ability of U0126 and LY294002 to induce apoptosis in MPM cells. DNA fragmentation analysis showed that treatment with U0126 or LY294002 alone induced apoptosis in EHMES-10 cells and in MSTO211H cells in a dose-dependent manner. Furthermore, the combined treatment with 80 µM U0126 and 80 µM LY294002 significantly increased the number of apoptotic cells compared to control and to treatment with U0126 alone in EHMES-10 cells (all, P<0.01) and compared to control and treatment with the individual drug in MSTO211H cells (all, P<0.01) (Fig. 2C). Western blot analysis showed that treatments with U0126 and/or LY294002 increased the level of 89 kDa cleaved PARP and decreased the levels of phospho-Bad and procaspase 3 in both cell lines (Fig. 2B).

Signaling alterations induced by treatment with U0126 and/or LY294002. To investigate the effects of U0126 and LY294002 on intercellular signaling, MPM cells were treated with U0126 or LY294002 or a combination of both. As shown in Fig. 2B, U0126 blocked the phosphorylation of ERK1/2 and p90RSK, and decreased HIF1α and VEGF expression in EHMES-10 cells. On the other hand, treatment with LY294002 suppressed the phosphorylation of Akt, p70S6K, S6 and GSK3β, and inhibited HIF1α and VEGF expression in EHMES-10 cells. Use of the combination treatment inhibited the phosphorylation of all of the above proteins and decreased the level of HIF1α and VEGF in EHMES-10 cells. Signaling alterations in MSTO211H cells tended to be similar to those in EHMES-10 cells, except for phospho-GSK3β alteration.

Antitumor activity of U0126 and/or LY294002 in EHMES-10 cell xenografts. To assess the in vivo therapeutic efficacy of U0126 and/or LY294002, SCID mice bearing EHMES-10 xenografts were treated with vehicle, U0126, LY294002, or a combination of U0126 and LY294002 as described in Materials and methods. Administration of 30 or 40 mg/kg of U0126, or 50 mg/kg of LY294002, or use of combined therapy with 30 mg/kg of U0126 and 25 mg/kg of LY294002 significantly prolonged the survival time of EHMES-10 cell-bearing SCID mice compared to the control group (all, P<0.01) (Fig. 3A-C). The combination therapy more effectively prolonged the survival time compared to treatment with either individual drug, although statistical significance was not obtained (Fig. 3C).

We also evaluated the effect of U0126 and/or LY294002 on the production of thoracic tumors and pleural effusion in...
EHMES-10 cell-bearing SCID mice (Fig. 3D, Table I). U0126 and/or LY294002 significantly inhibited tumor growth and pleural effusion production compared to control (all, \( P<0.05 \)). The combination therapy more effectively inhibited tumor growth compared to treatments with individual drugs, although statistical significance was not obtained.
Immunohistochemical staining and western blot analysis to clarify the antitumor mechanisms of U0126 and/or LY294002 in vivo. Immunohistochemical analysis showed that treatment with U0126 or with LY294002 reduced phospho-ERK1/2-positive tumor cells or phospho-Akt-positive tumor cells, respectively (Fig. 4A). Furthermore, treatment with an individual drug increased the number of p27kip1-positive tumor cells and TUNEL assay-positive tumor cells, and decreased the number of CD31-positive endothelial cells. The combination therapy group showed decreased phospho-ERK1/2 and phospho-Akt activities and CD31-positive endothelial cells, and increased p27kip1-positive tumor cells and TUNEL assay-positive tumor cells.
cells. The effect of the inhibitors delivered in combination was more pronounced than the drugs applied individually in the analyses of p27kip1 and TUNEL assay.

Western blot analysis showed that treatment with U0126, LY294002 and a combination of these agents inhibited ERK1/2 phosphorylation, Akt phosphorylation, and the phosphorylation of ERK1/2 and Akt, respectively (Fig. 4B). In treatment with U0126, LY294002 and the combination, we observed inhibited expression of cyclin E, cyclin D1, procaspase 3, HIF1α, VEGF, and CD31, and increased expression of p27kip1 and 89 kDa cleaved PARP.

Side effects of treatment with U0126 and/or LY294002. To examine the side effects of treatment with U0126 and/or LY294002, the body weights and serum levels of TP, BUN, Cre, AST and ALT were determined at the end of therapy on day 30 (Table II). Dermatopathy was also evaluated during the treatment with these agents. Side effects were not observed after the administration of these agents.

Discussion

The Ras pathway is one of the most frequently deregulated pathways in cancer (22). Ras signals through multiple effector pathways, including the RAF/MEK/ERK and PI3K/Akt signaling cascades. A previous study reported that these pathways were frequently activated in MPM (13). Therefore, downregulation of these pathways might contribute to the inhibition of tumor development and progression. In this study, we showed that treatment with MEK and PI3K inhibitors, U0126 and LY294002, inhibited MPM cell growth via cell cycle arrest, apoptosis, and inhibition of tumor angiogenesis in vitro and in vivo. In addition, each drug prolonged the survival time of SCID mice bearing EHMES-10 cells. When drugs were applied in combination, survival times were longer than those achieved with the individual treatments.

Treatment with a MEK inhibitor or a PI3K inhibitor has been shown to inhibit the growth of many types of cancer cells, including MPM cell lines, via induction of cell cycle arrest and apoptosis (14,15,23-25). In the present study, MEK and PI3K inhibitors suppressed growth of MPM cells in a dose-dependent manner. Flow cytometric analysis showed that the treatment with MEK or PI3K inhibitors achieved G1 cell cycle arrest of MPM cells. DNA fragmentation analysis showed apoptosis of MPM cells following treatment with these agents. It was reported that treatment of MPM with a MEK inhibitor induced p27kip1 upregulation (26). A PI3K inhibitor induced p27kip1 upregulation and inhibition of phosphorylation of p70S6K and S6 (14,26). The present study showed similar results. In addition, treatment of MPM cells with a MEK inhibitor downregulated cyclin E and cyclin D1, and inhibited phosphorylation of p90RSK and Bad. Inhibition of cyclin E and phospho-Bad expression was observed in treatment with a PI3K inhibitor for MPM cells. Treatment of EHMES-10 cells with a PI3K inhibitor reduced cyclin D1 and phospho-GSK3β expression.

Our study demonstrated the efficacy and the mechanisms of action of a MEK inhibitor and a PI3K inhibitor for MPM not only in vitro but also in vivo experiments. All previous reports of MEK inhibitors and PI3K inhibitors for MPM cells have been limited to showing the inhibitory effects and mechanisms in vitro (14,26). Our study showed that treatment with a MEK inhibitor or a PI3K inhibitor prolonged the survival time of EHMES-10 cells-bearing SCID mice. Tumor weight and pleural effusion at day 30 were reduced by these treatments. Furthermore, the immunohistochemical and western blot analyses of thoracic tumors suggested that the MEK and the PI3K inhibitors induced cell cycle arrest and cell apoptosis, which was compatible with the results of the in vitro study.

Treatments with the MEK inhibitor or the PI3K inhibitor might be associated with inhibition of angiogenesis in MPM cells. More than 60% of patients with MPM commonly present with a pleural effusion associated with breathlessness, often accompanied by chest wall pain, which compromises their quality of life (27). Angiogenesis has significant effects on the development of a pleural effusion and ascites (28,29). It was also reported that treatment with a MEK or a PI3K inhibitor suppressed proangiogenic cytokine production in melanoma and MPM cells (15,23). Our study showed that these agents significantly inhibited pleural effusion production and CD31 protein expression and decreased CD31-positive endothelial cells compared to controls. In addition, western blot analysis showed that treatment of these agents decreased the expression of HIF1α and VEGF, both of which play an essential role in tumor angiogenesis and progression, in vitro and in vivo.

Combination therapy with the MEK and PI3K inhibitors might be more rational than an individual drug for MPM. It was reported that antitumor activity of a MEK inhibitor or a PI3K inhibitor induces activation of the other pathway (30). Moreover, several reports demonstrated that inhibition of both cascades results in greater antitumor activity (15,23). In the present study, the combination therapy with MEK and PI3K inhibitors was also more effective compared to that of individual drugs both in vitro and in vivo.

Treatment with a MEK inhibitor and a PI3K inhibitor might be well tolerated. For example, treatment with the MEK inhibitor CI-1040 caused only mild or moderate toxicities such as diarrhea, nausea, asthenia, rash, and anorexia in patients with advanced non-small cell lung, breast, colon, and pancreatic cancers (31). In addition, Hu et al reported that daily intraperitoneal administration of LY294002 at a dose of 100 mg/kg caused body weight loss and dry skin in mice with ovarian cancer (32). However, in the following study, side effects were not shown by reduction of LY294002 administration to three days per week (33). In the present study, side effects were not observed using a combination of MEK and PI3K inhibitors.

In conclusion, our study demonstrates that in MPM cells our selected MEK and PI3K inhibitors functioned via cell cycle arrest, induction of apoptosis, and inhibition of tumor angiogenesis, both in vivo and in vitro. In addition, combining the MEK inhibitor with the PI3K inhibitor had additive or synergistic effects in vitro. Combination therapy with MEK and PI3K inhibitors may represent a promising novel therapeutic strategy in the treatment of MPM.

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