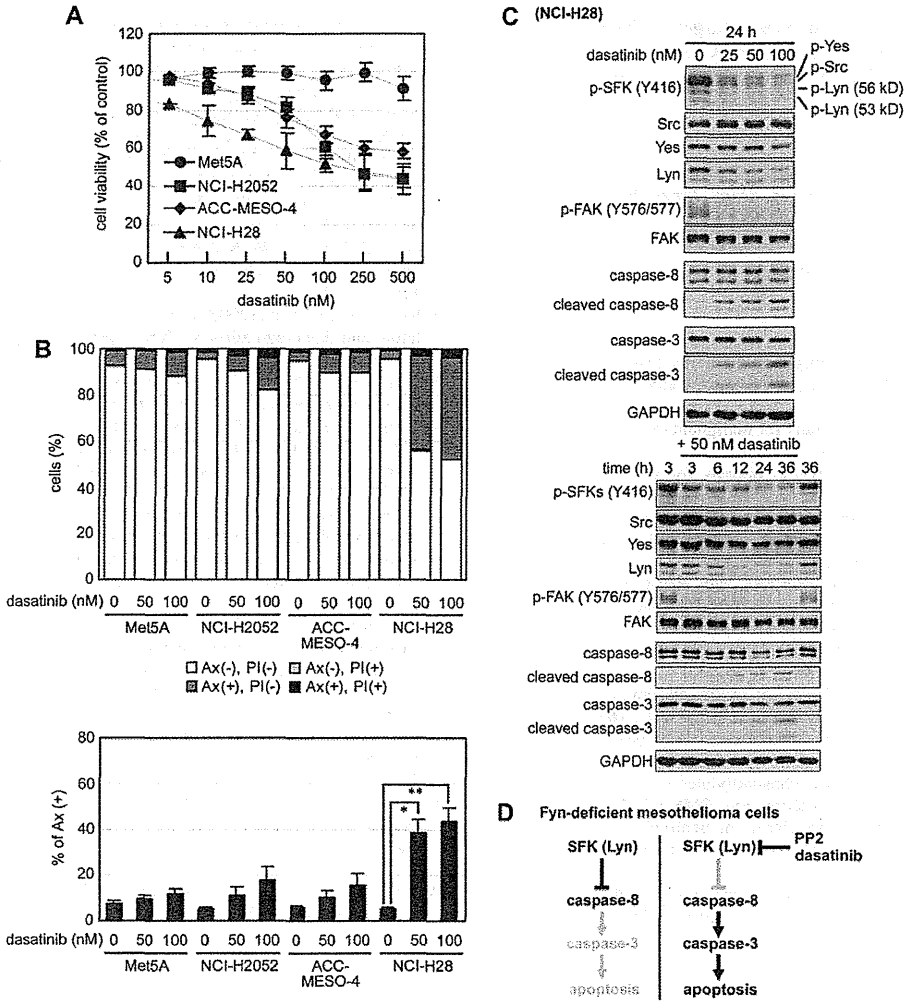

Fig. 6. SFK inhibitors induce reduction of Lyn protein followed by caspase-8-dependent apoptosis in Fyn-deficient mesothelioma cells. (**A**) Dasatinib suppresses cell viability of NCI-H2052, ACC-MESO-4 and NCI-H28 cells more markedly than that of Met5A cells. These cell lines were treated with dasatinib for 48 h at the indicated concentrations. Cell viability was assessed using the Cell Counting Kit-8 assay. (**B**) Dasatinib induces apoptosis in NCI-H28 cells more significantly than that in Met5A cells. Met5A, NCI-H2052, ACC-MESO-4 and NCI-H28 cells were treated with 50 and 100 nM dasatinib for 72 h and analyzed for Ax (+) apoptotic and propidium iodide (PI) (+) positive cells by flow cytometry. (**C**) Dasatinib inhibits SFK activity and reduces Lyn protein levels and caspase-8 activation in NCI-H28 cells. Cell extracts were prepared from NCI-H28 cells treated with dasatinib for 3–36 h at the indicated concentrations. (**D**) Schematic mechanism of apoptosis induced by SFK inhibitors in Fyn expression-suppressed mesothelioma cells. In Fyn expression-suppressed mesothelioma cells, SFK, in particular Lyn, suppresses caspase-8 activation (left panel), whereas SFK inhibitors, PP2 and dasatinib, reduce Lyn protein and apoptosis mediated by caspase-8 (right panel).

and Lyn-knockdown failed to induce apoptosis in other mesothelioma cell lines expressing Fyn (Figures 2, 3 and 5). Intriguingly, double knockdown of Fyn and Lyn induced caspase-8 activation and apoptosis in the mesothelioma cell lines expressing Fyn (Figure 5). These results suggest that Lyn plays a pivotal role in apoptosis of Fyn-deficient mesothelioma cells. We need to further investigate how Lyn and Fyn regulate apoptosis of mesothelioma cells.

Dasatinib is a highly potent Bcr-Abl kinase inhibitor used for the treatment of imatinib-resistant chronic myeloid leukemia (23). Dasatinib also inhibits SFK activity, leading to induction of apoptosis and cell cycle arrest and suppression of cell migration and invasion in mesothelioma cells (24). However, whether the apoptotic mechanism of dasatinib is the same as that of PP2 was not clear. We found that dasatinib inhibited SFK activity, reduced Lyn protein and induced apoptosis mediated by caspase-8 in NCI-H28 cells in a similar manner as PP2 (Figure 6). Recently, it has been reported that Src and Fyn are relevant targets for dasatinib action in lung cancer and that Lyn is a mediator of epithelial–mesenchymal transition and a target of dasatinib in breast cancer (25,26). These results suggest that dasatinib may be an effective molecular target drug against Fyn-deficient mesothelioma cells.

Preferential susceptibility to kinase inhibitor in NCI-H28 cells is also reported in c-Met, a receptor tyrosine kinase of hepatocyte growth factor, which is involved in cell growth, survival and migration (27). It has been shown that c-Met is overexpressed in NCI-H28 cells and that SU11274, a c-Met inhibitor, suppresses cell growth in NCI-H28 cells. If SU11274 induces reduction of Lyn protein in NCI-H28 cells, c-Met inhibitors as well as SFK inhibitors may induce apoptosis in Fyn-deficient mesothelioma cells.

In conclusion, SFK inhibitors, PP2 and dasatinib, reduce Lyn protein, leading to apoptosis mediated by caspase-8 in Fyn-deficient mesothelioma cells (Figure 6D). This study also shows potential utility of SFK inhibitors in the treatment of malignant mesothelioma and for the first time that deficiency of Fyn protein is prerequisite for apoptosis induced by SFK inhibitors in mesothelioma cells. Deficiency of Fyn protein would be a biomarker that discriminate the

sensitivity of SFK inhibitors in molecular-targeted therapy to mesothelioma. Additionally, further studies may open a novel way to develop therapy with both Fyn-deficiency and Lyn inhibition for this malignancy and other human tumor.

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Conflict of Interest Statement: None declared.

References

1. Robinson,B.W. *et al.* (2005) Malignant mesothelioma. *Lancet*, **366**, 397–408.
2. Tsao,A.S. *et al.* (2009) Malignant pleural mesothelioma. *J. Clin. Oncol.*, **27**, 2081–2090.
3. Vogelzang,N.J. (2008) Chemotherapy for malignant pleural mesothelioma. *Lancet*, **371**, 1640–1642.
4. Goudar,R.K. (2005) New therapeutic options for mesothelioma. *Curr. Oncol. Rep.*, **7**, 260–265.
5. Vogelzang,N.J. *et al.* (2003) Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. *J. Clin. Oncol.*, **21**, 2636–2644.
6. Yeatman,T.J. (2004) A renaissance for SRC. *Nat. Rev. Cancer*, **4**, 470–480.
7. Thomas,S.M. *et al.* (1997) Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol.*, **13**, 513–609.
8. Mitra,S.K. *et al.* (2005) Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell Biol.*, **6**, 56–68.
9. Schlaepfer,D.D. *et al.* (2004) Control of motile and invasive cell phenotypes by focal adhesion kinase. *Biochim. Biophys. Acta*, **1692**, 77–102.
10. Cohen,G.M. (1997) Caspases: the executioners of apoptosis. *Biochem. J.*, **326**, 1–16.
11. Cursi,S. *et al.* (2006) Src kinase phosphorylates Caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. *EMBO J.*, **25**, 1895–1905.
12. Jia,S.H. *et al.* (2008) Dynamic regulation of neutrophil survival through tyrosine phosphorylation or dephosphorylation of caspase-8. *J. Biol. Chem.*, **283**, 5402–5413.
13. Eguchi,R. *et al.* (2011) Arsenic trioxide induces apoptosis through JNK and ERK in human mesothelioma cells. *J. Cell. Physiol.*, **226**, 762–768.
14. Ingley,E. *et al.* (2006) Csk-binding protein mediates sequential enzymatic down-regulation and degradation of Lyn in erythropoietin-stimulated cells. *J. Biol. Chem.*, **281**, 31920–31929.
15. Lee,M. *et al.* (2004) Apoptotic effect of PP2 a Src tyrosine kinase inhibitor, in murine B cell leukemia. *J. Cell. Biochem.*, **93**, 629–638.
16. Liu,X. *et al.* (2010) The role of enoyl-CoA hydratase short chain 1 and peroxiredoxin 3 in PP2-induced apoptosis in human breast cancer MCF-7 cells. *FEBS Lett.*, **584**, 3185–3192.
17. De Toni,E.N. *et al.* (2007) P60-c-src suppresses apoptosis through inhibition of caspase 8 activation in hepatoma cells, but not in primary hepatocytes. *J. Hepatol.*, **46**, 682–691.
18. Saito,Y.D. *et al.* (2009) Fyn: a novel molecular target in cancer. *Cancer*, **116**, 1629–1637.
19. Sørensen,K.D. *et al.* (2008) Chromosomal deletion, promoter hypermethylation and downregulation of FYN in prostate cancer. *Int. J. Cancer*, **122**, 509–519.
20. Ban,K. *et al.* (2008) BCR-ABL1 mediates up-regulation of Fyn in chronic myelogenous leukemia. *Blood*, **111**, 2904–2908.
21. Grosso,S. *et al.* (2009) Gene expression profiling of imatinib and PD166326-resistant CML cell lines identifies Fyn as a gene associated with resistance to BCR-ABL inhibitors. *Mol. Cancer Ther.*, **8**, 1924–1933.
22. Scapini,P. *et al.* (2009) Multiple roles of Lyn kinase in myeloid cell signaling and function. *Immunol. Rev.*, **228**, 23–40.
23. Khoury,H.J. *et al.* (2009) Dasatinib treatment for Philadelphia chromosome-positive leukemias: practical considerations. *Cancer*, **115**, 1381–1394.
24. Tsao,A.S. *et al.* (2007) Inhibition of c-Src expression and activation in malignant pleural mesothelioma tissues leads to apoptosis, cell cycle arrest, and decreased migration and invasion. *Mol. Cancer Ther.*, **6**, 1962–1972.
25. Li,J. *et al.* (2010) A chemical and phosphoproteomic characterization of dasatinib action in lung cancer. *Nat. Chem. Biol.*, **6**, 291–299.
26. Choi,Y.-L.a *et al.* (2010) Lyn is a mediator of epithelial-mesenchymal transition and a target of dasatinib in breast cancer. *Cancer Res.*, **70**, 2296–2306.
27. Jagadeeswaran,R. *et al.* (2006) Functional analysis of c-Met/hepatocyte growth factor pathway in malignant pleural mesothelioma. *Cancer Res.*, **66**, 352–361.

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Gene Therapy Using Adenovirus Against Malignant Mesothelioma

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Abstract. *Background: Adenovirus vectors have been utilized for cancer gene therapies. The present study examined the oncolytic effects of adenovirus type 5 (Ad5) and fiber-substituted conditionally replicating adenovirus (CRAD) Ad5/F35 vectors on the human malignant mesothelioma cells MSTO-211H, NCI-H28, NCI-H2052, and NCI-H2452 cells. Materials and Method: For the adenovirus, the first mRNA/protein to be made (~1 h after infection) is E1A. Ad5F35 and Ad5 CRAD vectors containing the E1 gene controlled by the human midkine promoter (Ad5F35/MKp-E1 and Ad5/MKp-E1, respectively) were constructed. Western blotting and cell viability assays were carried out in cells transfected with Ad5/MKp-E1 and Ad5F35/MKp-E1. Results: Coxsackie and adenovirus receptor (CAR), a cell surface target of Ad5, and CD46, a cell surface target of Ad35, were expressed in all the malignant mesothelioma cell lines examined here, as much as in HEK293 cells, with no significant differences in the expression levels among cells. Both Ad5/MKp-E1 and Ad5F35/MKp-E1 induced oncolysis of malignant mesothelioma cells in a viral particle-dependent manner, with similar efficacy. Conclusion: The results of the present study suggest that both Ad5/MKp-E1 and Ad5F35/MKp-E1 are useful for the gene therapy of human malignant mesothelioma.*

Malignant mesothelioma is an aggressive and highly lethal tumor which is caused by occupational exposure to asbestos fibers, particularly of the amphibole type (1). In spite of great efforts, no promising treatment has been provided as yet for malignant mesothelioma. Establishing new effective therapies for malignant mesothelioma, therefore, is of great importance.

Recent studies have focused upon gene therapies using adenovirus vectors for a variety of cancer types (2). The critical key point for the gene therapies is the quantity of adenovirus, which is transferred into tumor cells through adenovirus-targeting receptors (3, 4). Another key point for gene therapy is to reduce side-effects. To address this point, conditionally replicating adenovirus (CRAD) has been developed (5, 6). CRAD is capable of inducing tumor-specific cell death and of amplifying oncolysis due to intratumoral replication (7). CRAD is also capable of inducing oncolysis of cancer cells neighboring primarily infected cells by secondary infection, to an extent greater than that for non-replicating adenoviruses (8-10).

For renal cell carcinoma (RCC), an adenovirus vector containing an Arg-Gly-Asp (RGD) motif has been shown to increase the efficacy of gene transfer into cells (11). Adenovirus type 5 (Ad5) has been widely used for gene therapies. Ad5 is infected into cells through coxsackie and adenovirus receptor (CAR) (12). CAR expression, however, is low in some types of cancer cell (13). In contrast, CD46, a cell surface receptor for adenovirus type 35 (Ad35), is more commonly expressed in cancer cells (14).

For the adenovirus, the first mRNA/protein to be made (~1 h after infection) is E1A. In the present study, we constructed a CRAD vector encoding the *E1* gene under the control of a 0.6-kb midkine promoter without (Ad5/MKp-E1) and with replacement of the fiber knob for Ad5 by that for Ad35 (Ad5F35/MKp-E1), and examined their oncolytic effect on human malignant mesothelioma cells. We show here that both the Ad5/MKp-E1 and Ad5F35/MKp-E1 are suitable for gene therapy of human malignant mesothelioma.

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Key Words: Adenovirus, oncolysis, CAR, CD46, malignant mesothelioma.

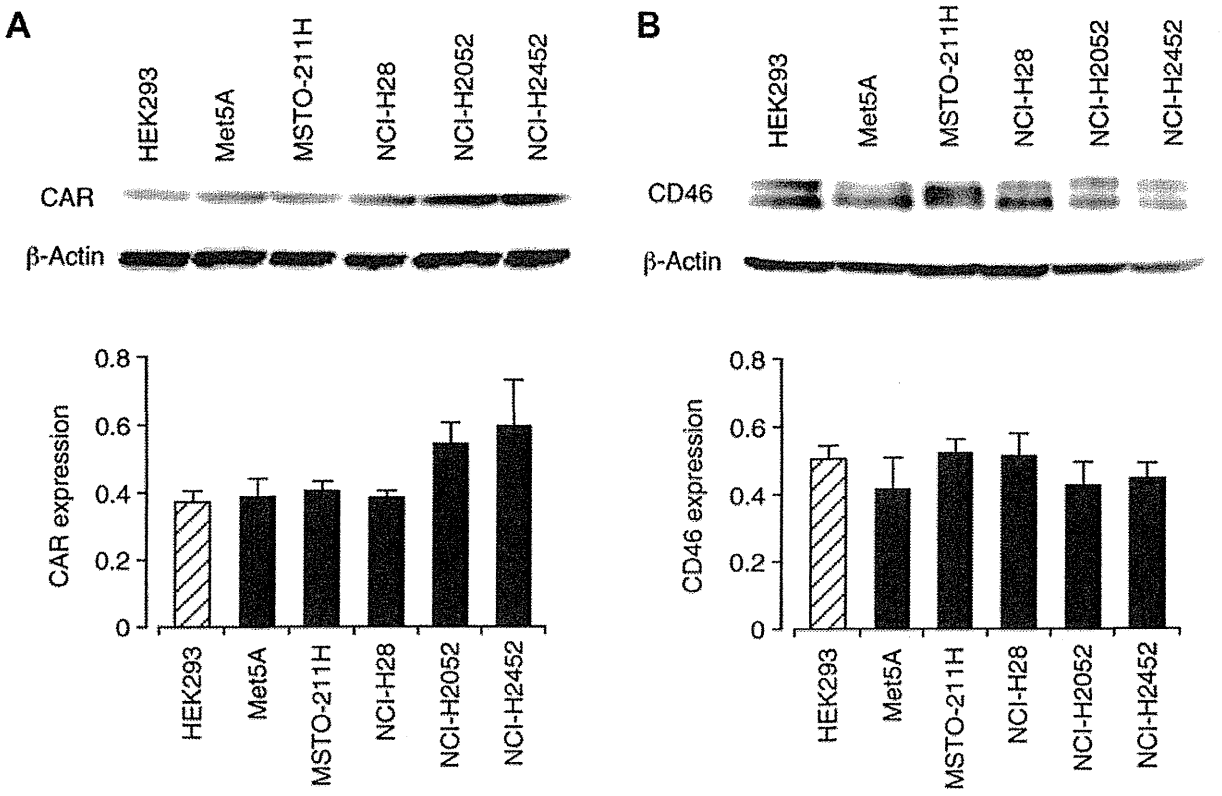

Figure 1. *Expression of CAR and CD46 protein. Western blotting for CAR (A) and CD46 (B) were carried out in cells, as indicated. The expression levels of each protein were normalized to those for β-actin. In the graphs, each column represents the mean (±SEM) expression of each protein (n=4 independent experiments).*

Materials and Methods

Construction of CRAD vectors. Replication-competent adenovirus vector containing the E1 gene controlled by the midkine promoter was prepared as follows. The cytomegalovirus (CMV) promoter of pShuttle2 vector (Clontech Laboratories, Mountain View, CA, USA) was replaced with the 0.6-kb 5'-upstream regulatory region of the midkine gene and the pS-MK/E1 vector was produced by inserting the *E1* gene downstream of the midkine region. To construct pAd5F35, the Ad5 fiber region on the pAdeno-X vector (Clontech Laboratories) was substituted with the Ad35 fiber region on the RHSP vector (Avior Therapeutics, Seattle, WA, USA). Ad5/MKp-E1 and Ad5F35/MKp-E1 were prepared by linking the pS-MK/E1 vector with pAdeno-X and pAd5F35, respectively.

Cell culture. Human malignant pleural mesothelioma cell lines MSTO-211H, NCI-H28, NCI-H2052 and NCI-H2452, and Met5A human mesothelial cells, were purchased from the American Type Culture Collection (Manassas, VA, USA) and HEK293 cells from RIKEN Bioresource Center (Tsukuba, Japan). Malignant mesothelioma and mesothelial cells were grown in RPMI-1640 medium and HEK293 cells in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.003% L-glutamine, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Western blotting. Western blotting was carried out by a method, as described previously (15). Briefly, cells were lysed with 1% (w/v) sodium dodecyl sulfate (SDS). Proteins were then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a TGX gel (BioRad, Hercules, CA, USA) and then transferred to polyvinylidene difluoride membranes. Membranes were then blocked with TBS-T [150 mM NaCl, 0.1% (v/v) Tween20 and 20 mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin and subsequently incubated with antibodies against CAR (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD46 (Santa Cruz Biotechnology), and β-actin (Sigma, St Louis, MO, USA). Immunoreactivity was detected with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and visualized using a chemiluminescence detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). Signal intensities for CAR or CD46 protein were normalized by the intensity for β-actin.

Oncolysis assay. Cells (5×10^3) in 96-well plates were infected with Ad5/MKp-E1 or Ad5F35/MKp-E1 at 0.001-10000 viral particle (VP)/cells. Five days after infection, cells were incubated with Alamar Blue (Life Technologies, Gaithersburg, MD, USA) and the

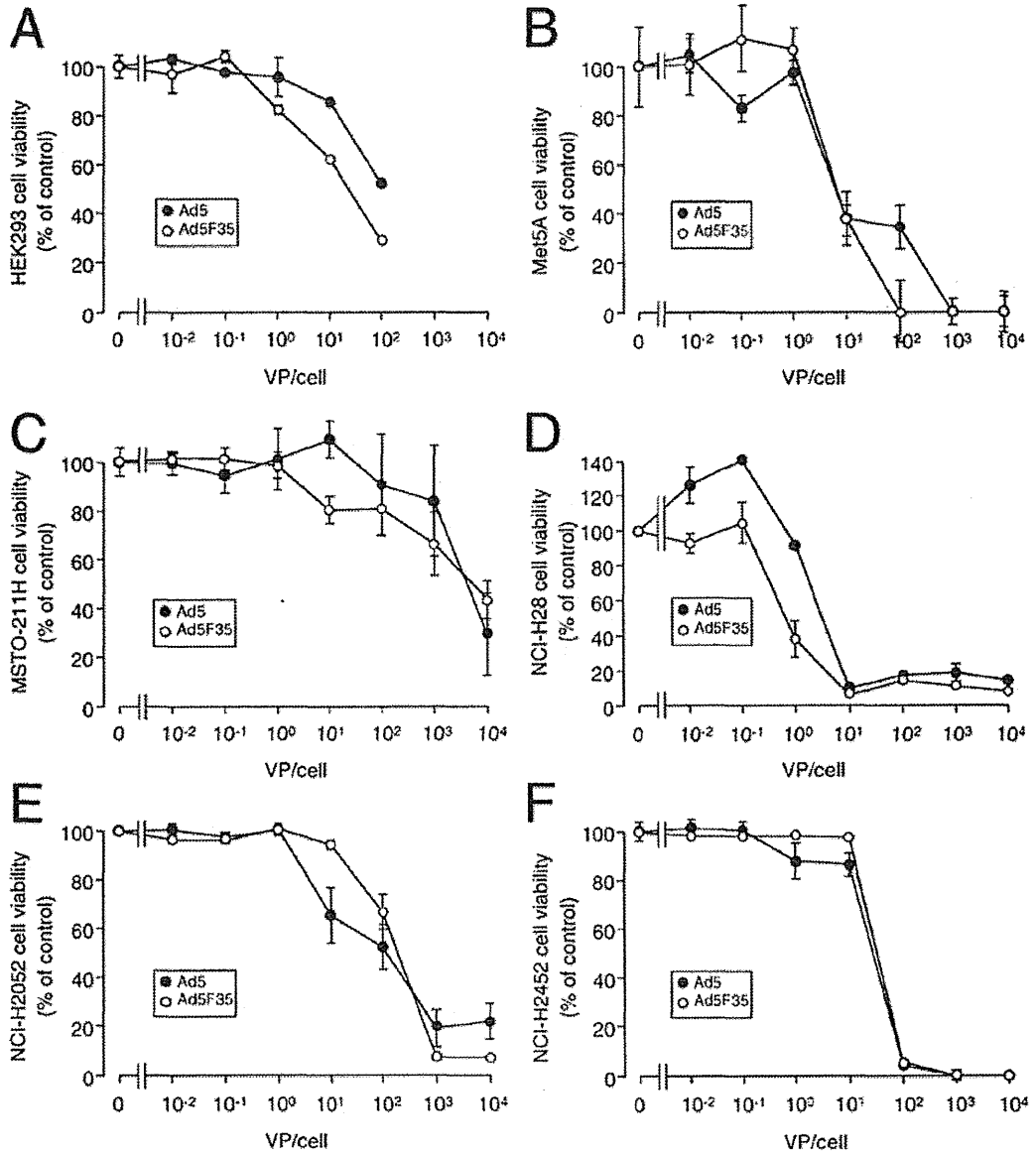

Figure 2. *Oncolytic effects of Ad5/MKp-E1 and Ad5F35/MKp-E1. Cell viability was assayed 5 days after infection using an Alamar Blue in HEK293 (A), Met5A (B), MSTO-211H (C), NCI-H28 (D), NCI-H2052 (E), and NCI-H2452 cells (F). In the graphs, each point represents the mean (±SEM) percentage of basal levels (viabilities of cells uninfected) (n=6 independent experiments).*

number of viable cells were counted by detecting the absorbance at 560 nm with a high-throughput microplate spectrophotometer (SpectraMax Plus 384; Molecular Devices, Sunnyvale, CA, USA). Viabilities for cells without infection with Ad5/MKp-E1 or Ad5F35/MKp-E1 were regarded as controls.

Results

Expression of CAR and CD46. As previously demonstrated (16), HEK293 cells abundantly express CAR and CD46 (Figure 1A and B). Met5A mesothelial cells and all the malignant mesothelioma cells examined here expressed CAR and CD46, to an extent similar to that of HEK293 cells (Figure 1A and B).

Oncolytic effects of Ad5/MKp-E1 and Ad5F35/MKp-E1. For HEK293 cells, Ad5/MKp-E1 and Ad5F35/MKp-E1 reduced cell viability in a VP-dependent manner, with Ad5F35/MKp-E1 efficacy being slightly higher than that of Ad5/MKp-E1

(Figure 2A). Ad5/MKp-E1 and Ad5F35/MKp-E1 also reduced cell viability in a VP-dependent manner for the Met5A mesothelial cells and all the malignant mesothelioma cells, with no difference in the efficacy between Ad5/MKp-E1 and Ad5F35/MKp-E1 (Figure 2B-F).

Discussion

Midkine is a heparin-binding growth factor that is induced by retinoic acid in embryonal carcinoma cells (17). It is implicated in mitogenesis, angiogenesis, anti-apoptosis, fibrinolysis, and transformation (18-22). Midkine is enriched in a variety of cancer cells originating from the esophagus, stomach, colon, liver, breast, and pancreas, while its expression in non-malignant cells is quite limited, with moderate expression in the kidney and weak expression in the lung, colon, and thyroid gland (23-27). The midkine promoter, accordingly, could be utilized for suicide gene therapy.

We, therefore, constructed Ad5 (Ad5/MKp-E1) and Ad5F35 CRAD vectors (Ad5F35/MKp-E1) encoding the *E1* gene under the control of a 0.6-kb midkine promoter. Ad5 and Ad35 are infected into cells through CAR and CD46 (12, 14). CD46 is recognized to be more commonly expressed in cancer cells as compared with CAR (13). In the present study, however, CAR as well as CD46 were abundantly expressed in malignant mesothelioma cells. Both Ad5/MKp-E1 and Ad5F35/MKp-E1 exhibited sufficient oncolytic effect on all the malignant mesothelioma cell lines examined here, with no difference in the efficacy between Ad5/MKp-E1 and Ad5F35/MKp-E1. Ad5/MKp-E1 and Ad5F35/MKp-E1, thus, appear to be suitable for gene therapy of human malignant mesothelioma.

Conclusion

The results of the present study show that a sufficient and beneficial oncolytic effect on malignant mesothelioma cells is obtained with Ad5/MKp-E1 and Ad5F35/MKp-E1. These adenovirus vectors could be developed as a promising gene therapy option for malignant mesothelioma.

Potential Conflict of Interest Statement

None of the Authors have any potential conflict of interest.

References

1 Tsujimura T, Torii I, Sato A, Song M, Fukuoka K, Hasegawa S and Nakano T: Pathological and molecular biological approaches to early mesothelioma. Int J Clin Oncol *17*: 40-47, 2012.

2 Guse K, Cerullo V and Hemminki A: Oncolytic Vaccinia virus for the treatment of cancer. Expert Opin Biol Ther *11*: 595-608, 2011.

3 Bauerschmitz GJ, Barker SD and Hemminki A: Adenoviral gene therapy for cancer: From vectors to targeted and replication competent agents (review). Int J Oncol *21*: 1161-1174, 2002.

4 Kanerva A and Hemminki A: Modified adenoviruses for cancer gene therapy. Int J Cancer *110*: 475-480, 2004.

5 Ganly I, Kirn D, Eckhardt G, Rodriguez GI, Soutar DS, Otto R, Robertson AG, Park O, Gulley ML, Heise C, Von Hoff DD and Kaye SB: A phase I study of Onyx-015, an E1B-attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. Clin Cancer Res *6*: 798-806, 2000.

6 Kirn D: Oncolytic virotherapy for cancer with the adenovirus dl1520 (Onyx-015): Results of phase I and II trials. Expert Opin Biol Ther *1*: 525-538, 2001.

7 Biederer C, Ries S, Brandts CH and McCormick F: Replication-selective viruses for cancer therapy. J Mol Med (Berl) 80: 163-175, 2002.

8 Alemany R, Balagué C and Curiel DT: Replicative adenoviruses for cancer therapy. Nat Biotechnol *18*: 723-727, 2000.

9 Heise C and Kirn DH: Replication-selective adenoviruses as oncolytic agents. J Clin Invest *105*: 847-851, 2000.

10 Ring CJ: Cytolytic viruses as potential anticancer agents. J Gen Virol *83*: 491-502, 2002.

11 Terao S, Acharya B, Suzuki T, Aoi T, Naoe M, Hamada K, Mizuguchi H and Gotoh A: Improved gene transfer into renal carcinoma cells using adenovirus vector containing RGD motif. Anticancer Res *29*: 2997-3001, 2009.

12 Hamilton MM, Byrnes GA, Gall JG, Brough DE, King CR and Wei LL: Alternate serotype adenovector provides long-term therapeutic gene expression in the eye. Mol Vis *14*: 2535-2546, 2008.

13 Okegawa T, Sayne JR, Nutahara K, Pong RC, Saboorian H, Kabbani W, Higashihara E and Hsieh JT: A histone deacetylase inhibitor enhances adenoviral infection of renal cancer cells. J Urol *177*: 1148-1156, 2007.

14 Gaggar A, Shayakhmetov DM and Lieber A: CD46 is a cellular receptor for group B adenoviruses. Nat Med 9: 1408-1412, 2003.

15 Kamiya H, Kanno T, Fujita Y, Gotoh A, Nakano T and Nishizaki T: Apoptosis-related gene transcription in human A549 lung cancer cells *via* A_3 adenosine receptor. Cell Physiol Biochem *29*: 687-696, 2012.

16 Nagaya H, Tagawa M, Hiwasa K, Terao S, Kanno T, Nishizaki T and Gotoh A: Fiber-substituted conditionally replicating adenovirus for oncolysis of human renal carcinoma cells. Anticancer Res *32*: 2985-2989, 2012.

17 Kadomatsu K: Midkine, a heparin-binding growth factor: Its discovery and functions. Seikagaku *70*: 1315-1325, 1998 (in Japanese).

18 Mashour GA, Ratner N, Khan GA, Wang HL, Martuza RL and Kurtz A: The angiogenic factor midkine is aberrantly expressed in NF1-deficient Schwann cells and is a mitogen for neurofibroma-derived cells. Oncogene *20*: 97-105, 2001.

19 Choudhuri R, Zhang HT, Donnini S, Ziche M and Bicknell R: An angiogenic role for the neurokines midkine and pleiotrophin in tumorigenesis. Cancer Res *57*: 1814-1819, 1997.

20 Qi M, Ikematsu S, Ichihara-Tanaka K, Sakuma S, Muramatsu T and Kadomatsu K: Midkine rescues Wilms' tumor cells from cisplatin-induced apoptosis: Regulation of Bcl-2 expression by midkine. J Biochem *127*: 269-277, 2000.

21 Kojima S, Inui T, Muramatsu H, Kimura T, Sakakibara S and Muramatsu T: Midkine is a heat and acid stable polypeptide capable of enhancing plasminogen activator activity and neurite outgrowth extension. Biochem Biophys Res Commun *216*: 574-581, 1995.

22 Kadomatsu K, Hagihara M, Akhter S, Fan QW, Muramatsu H and Muramatsu T: Midkine induces the transformation of NIH3T3 cells. Br J Cancer *75*: 354-359, 1997.
23 Tsutsui J, Kadomatsu K, Matsubara S, Nakagawara A, Hamanoue M, Takao S, Shimazu H, Ohi Y and Muramatsu T: A new family of heparin-binding growth/differentiation factors: increased midkine expression in Wilms' tumor and other human carcinomas. Cancer Res *53*: 1281-1285, 1993.
24 Aridome K, Tsutsui J, Takao S, Kadomatsu K, Ozawa M, Aikou T and Muramatsu T: Increased midkine gene expression in human gastrointestinal cancers. Jpn J Cancer Res *86*: 655-661, 1995.
25 Garver RI Jr., Radford DM, Donis-Keller H, Wick MR and Milner PG: Midkine and pleiotrophin expression in normal and malignant breast tissue. Cancer *74*: 1584-1590, 1994.
26 Koide N, Hada H, Shinji T, Ujike K, Hirasaki S, Yumoto Y, Hanafusa T, Kadomatsu K, Muramatsu H, Muramatsu T and Tsuji T: Expression of the midkine gene in human hepatocellular carcinomas. Hepatogastroenterology *46*: 3189-3196, 1999.
27 Muramatsu T: Midkine (MK), the product of a retinoic acid responsive gene, and pleiotrophin constitute a new protein family regulating growth and differentiation. Int J Dev Biol *37*: 183-188, 1993.

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Original Paper

Sphingosine Suppresses Mesothelioma Cell Proliferation by Inhibiting PKC-δ and Inducing Cell Cycle Arrest at the G_0/G_1 Phase

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Key Words
Sphingosine • Protein kinase C-δ • Mesothelioma cell • Proliferation • Suppression

Abstract
Background/Aims: Sphingosine regulates cellular differentiation, cell growth, and apoptosis. The present study aimed at understanding sphingosine-regulated mesothelioma cell proliferation. ***Methods:*** Human malignant mesothelioma cells such as NCI-H28, NCI-H2052, NCI-H2452, and MSTO-211H cells were cultured. The siRNA to silence the protein kinase C (PKC)-δ-targeted gene was constructed and transfected into cells. MTT assay, cell cycle analysis using a flow cytometry, and cell-free PKC-δ assay were carried out. ***Results:*** For all the cell types sphingosine inhibited cell growth in a concentration (1-100 μM)-dependent manner. The sphingosine effect was not prevented by rottlerin, an inhibitor of protein kinase C-δ (PKC-δ); conversely, rottlerin further enhanced the sphingosine effect or rottlerin suppressed mesothelioma cell growth without sphingosine. In the cell-free PKC assay, sphingosine attenuated PKC-δ activity. Knocking-down PKC-δ induced cell cycle arrest at the G_0/G_1 phase and inhibited cell growth. ***Conclusion:*** The results of the present study show that sphingosine suppressed mesothelioma cell proliferation by inhibiting PKC-δ, to induce cell cycle arrest at the G_0/G_1 phase.

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Introduction

Sphingolipids include ceramide, ceramide 1-phosphate, sphingosine, and sphingosine 1-phosphate (S1P). Sphingosine is produced from ceramidase-catalyzed cleavage of fatty acids from ceramide. Evidence has pointed to the role of sphingolipid in the regulation of cellular differentiation, cell growth, and apoptosis. We have earlier found that sphingosine induces apoptosis in hippocampal neurons and astrocytes by activating caspase-3/-9 via a sphingosine-dependent protein kinase (SDK)/14-3-3 protein/Bax/cytochrome c pathway [1]. SDK is produced through proteolytic processing of protein kinase C-δ (PKC-δ) and activated by binding sphingosine [2]. SDK specifically phosphorylates 14-3-3 protein [3, 4], thereby dissociating Bax from a complex with 14-3-3 protein, to induce a mitochondria-mediated apoptosis. Sphingosine, alternatively, induces apoptosis in rhabdomyosarcoma cells by activating caspase-3/-9 in a Bax-dependent manner [5] or in mouse BALB/c 3T3 clone A31 cells in an SDK-dependent manner [2]. In our recent study, sphingosine induced apoptosis in well differentiated MKN-28 human gastric cancer cells by increasing SDK production from PKC-δ, to phosphorylate 14-3-3 protein, thereby causing disruption of mitochondrial membrane potentials and activating caspase-9 followed by the effector caspase-3. Sphingosine, thus, might be a target for development of anti-tumor drugs.

Malignant mesothelioma is an aggressive tumor arised from previous asbestos exposure. A great deal of challenge has been attempted, yet disappointingly, malignant mesothelioma is considerably resistant to conventional anticancer therapies and no beneficial effect is expected. Then, we were prompted to assess the effect of sphingosine on proliferation of malignant mesothelioma cells. We show here that sphingosine suppresses mesothelioma cell proliferation by inhibiting PKC-δ, to induce cell cycle arrest at the G_0/G_1.

Materials and Methods

Cell culture

NCI-H28, NCI-H2052, NCI-H2452, and MSTO-211H cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 0.003% (w/v) L-glutamine, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Assay of cell viability

Cell viability was assayed by the method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as described previously [1]. MTT-reactive cells were quantified at an absorbance of 570 nm using a micro-plate reader (SPECTRAmax PLUS384, Molecular Devices, Sunnyvale, CA, USA).

Cell cycle analysis

Cells were harvested by a trypsinization, fixed with 70% (v/v) ethanol at 4 °C overnight. Fixed cells were incubated in phosphate-buffered saline containing 1.5 μg/ml RNase A for 1 h at 37 °C, followed by staining with 5 μl of propidium iodide (PI) for 20 min on ice. Then, cells were collected on a nylon mesh filter (pore size, 40 μm), and cell cycles were assayed using a flowcytometer (FACSCalibur, Becton Dickinson, USA) at an excitation of 488 nm and an emission of 585 nm, and analyzed using FlowJo software (TreeStar, San Carlos, CA, USA).

Cell-free PKC-δ assay

PKC activity in the cell-free systems was quantified by the method as previously described [6]. Briefly, synthetic PKC substrate peptide (10 μM) was reacted with PKC-δ in a Ca^{2+}-free medium containing 20 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, and 10 μM ATP with and without sphingosine in the absence of phosphatidylserine and diacylglycerol at 30 °C for 5 min. After loading on a reversed phase high performance liquid chromatography (HPLC)(LC-10ATvp, Shimadzu Co., Kyoto, Japan), a substrate peptide peak and a new

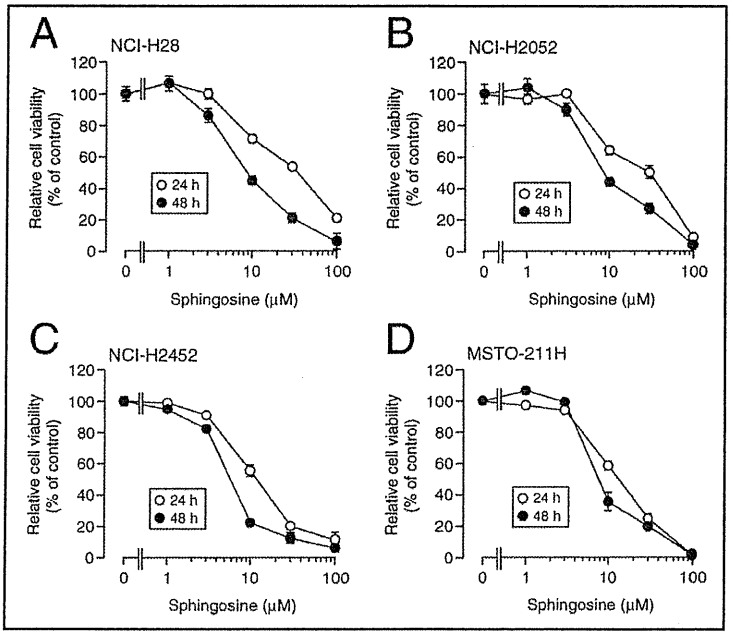

Fig. 1. Sphingosine-induced suppression of cell viability. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were treated with sphingosine at concentrations as indicated for 24-48 h, and then, MTT assay was carried out. In the graphs, each point represents the mean (± SEM) percentage of basal cell viabilities (MTT intensities before treatment with sphingosine)(n=4 independent experiments).

product peak were detected at an absorbance of 214 nm. Areas for non-phosphorylated and phosphorylated PKC substrate peptide were measured (total area corresponds to concentration of PKC substrate peptide used here), and the amount of phosphorylated substrate peptide was calculated. Phosphorylated substrate peptide (pmol/1 min) was used as an index of PKC-δ activity.

Construction and transfection of siRNA

The siRNA to silence PKC-δ-targeted gene (PKC-δ siRNA) and the negative control siRNA (NC siRNA) were obtained from BONAC (Fukuoka, Japan). The PKC-δ siRNA and the NC siRNA were reverse-transfected into cells using a Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). Cells were used for experiments 48 h after transfection.

Western blotting

Cells were lysed with 1% (w/v) sodium dodecyl sulfate (SDS). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a TGX gel (BioRad, Hercules, CA, USA) and then transferred to polyvinylidene difluoride membranes. Blotting membranes were blocked with TBS-T [150 mM NaCl, 0.1% (v/v) Tween20 and 20 mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin and subsequently incubated with an anti-PKC-δ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or an anti-β-actin antibody (Sigma, St Louis, MO, USA). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and visualized using a chemiluminescence detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Pierce, Rockford, IL, USA).

Statistical analysis

Statistical analysis was carried out using Fisher's Protected Least Significant Difference (PLSD) test, unpaired *t*-test, and Dunnett's test.

Results

Sphingosine suppresses mesothelioma cell proliferation

For all the mesothelioma cells examined here treatment with sphingosine for 24-48 h decreased the number of viable cells in a concentration (1-100 μM)-dependent manner,

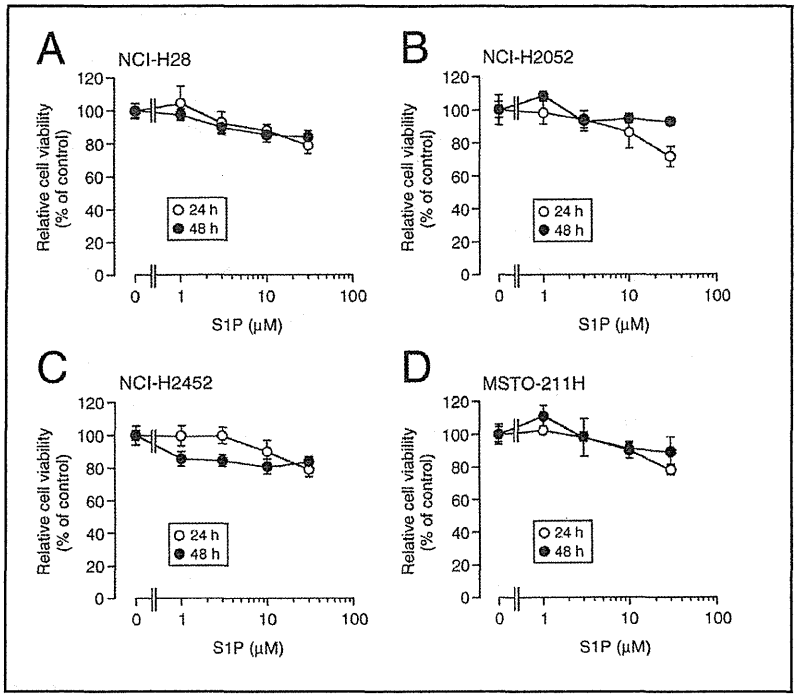

Fig. 2. The effect of S1P on cell viability. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were treated with S1P at concentrations as indicated for 24-48 h, and then, MTT assay was carried out. In the graphs, each point represents the mean (± SEM) percentage of basal cell viabilities (MTT intensities before treatment with S1P) (n=4 independent experiments).

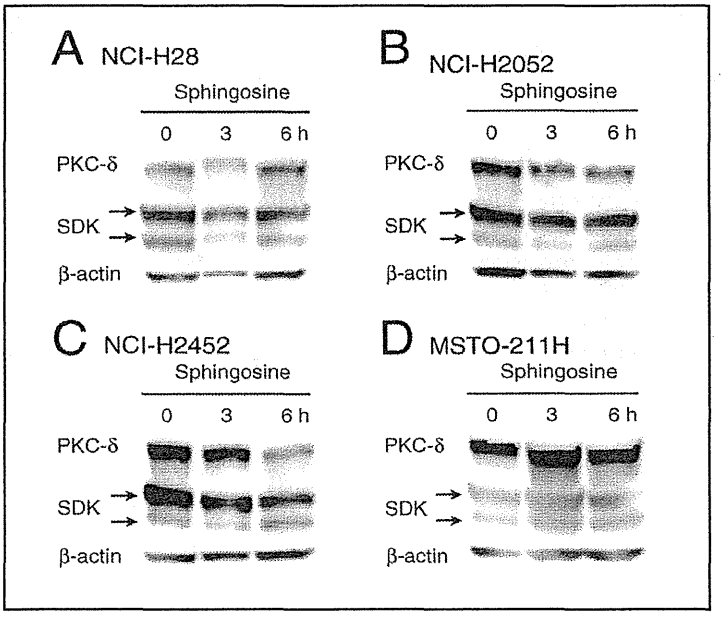

Fig. 3. The effect of sphingosine on SDK production. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were treated with sphingosine (100 μM) for 0-6 h, and Western blotting was carried out using an anti-PKC-δ antibody. Note that no increase in the SDK production (arrows) was found with sphingosine treatment and that similar results were obtained with 4 independent experiments.

without difference in the extent among the cell types (Fig. 1A, B, C, D). In the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, no significant increase in TUNEL-positive cells was obtained with sphingosine for all the cell types (data not shown). This suggests that sphingosine do not induce mesothelioma cell apoptosis but suppresses mesothelioma cell proliferation.

Sphingosine is phosphorylated by sphingosine kinase 1 to produce S1P. Treatment with S1P for 24-48 h had little effect on the number of viable cells at concentrations ranging from 1 to 30 μM for all the cell types (Fig. 2A, B, C, D). This indicates that sphingosine by itself, but not S1P produced from sphingosine, has the potential to suppress mesothelioma cell proliferation.

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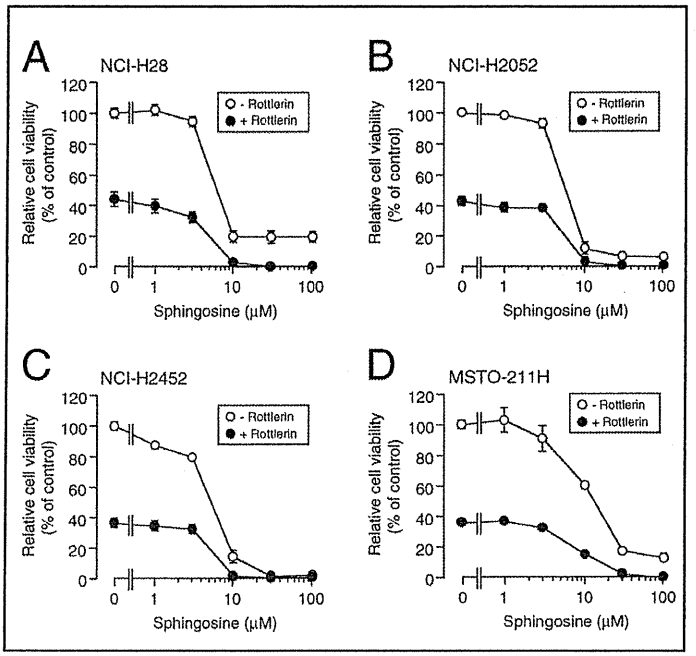

Fig. 4. The effect of rottlerin on sphingosine-induced suppression of cell viability. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were treated with sphingosine at concentrations as indicated in the presence and absence of rottlerin (10 μM) for 24 h, and then, MTT assay was carried out. In the graphs, each point represents the mean (± SEM) percentage of basal cell viabilities (MTT intensities before treatment with sphingosine in the absence of rottlerin)(n=4 independent experiments).

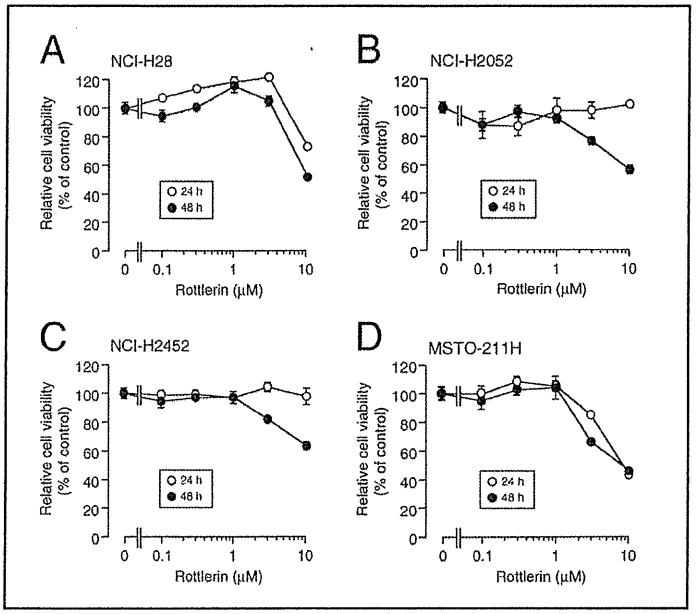

Fig. 5. Rottlerin-induced suppression of cell viability. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were treated with rottlerin alone at concentrations as indicated for 24-48 h, and then, MTT assay was carried out. In the graphs, each point represents the mean (± SEM) percentage of basal cell viabilities (MTT intensities before treatment with rottlerin) (n=4 independent experiments).

In our earlier study, sphingosine induced apoptosis in hippocampal neurons and astrocytes via a PKC-δ/SDK pathway [1]. We subsequently examined whether the sphingosine effect is due to PKC-δ/SDK activation. Sphingosine did not increase SDK production in all the cell types (Fig. 3A, B, C, D), ruling out the participation of SDK in the sphingosine effect here. Sphingosine-induced decrease in the number of viable cells was not inhibited by rottlerin (10 μM), an inhibitor of PKC-δ; conversely, further decrease in the number of viable cells was found in the presence of rottlerin ($P<0.001$ as compared with the effect of sphingosine in the absence of rottlerin for all the cell types, Fisher's PLSD test)(Fig. 4A, B, C, D). Moreover, treatment with rottlerin alone for 24-48 h decreased the number of viable cells in a concentration (0.1-10 μM)-dependent manner for all the cell types (Fig. 5A, B, C,

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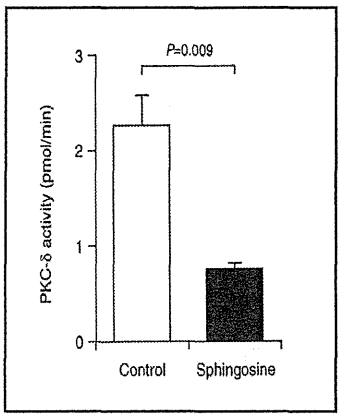

Fig. 6. Sphingosine-induced PKC-δ inhibition. In the cell-free systems, PKC-δ activity was assayed in the presence and absence of sphingosine (100 μM). In the graph, each column represents the mean (± SEM) PKC-δ activity (pmol/min)(n=4). *P* value, unpaired *t*-test.

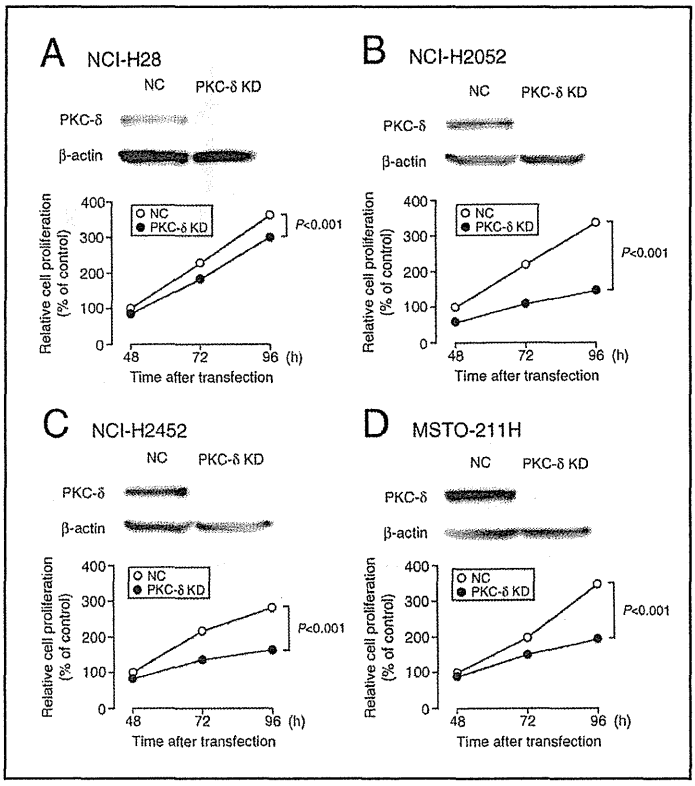

Fig. 7. The effect of PKC-δ knock-down on cell viability. MTT assay was carried out in NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) transfected with the NC siRNA (NC) or the PKC-δ siRNA (PKC-δ KD). Note that expression of PKC-δ is drastically decreased in all the cell types transfected with the PKC-δ siRNA in the Western blot analysis. In the graphs, each point represents the mean (± SEM) percentage of basal cell viabilities (MTT intensities for cells transfected with NC siRNA at 48 h after transfection)(n=4 independent experiments). *P* values, Fisher's PLSD test.

D). Taken together, these results suggest that PKC-δ promotes proliferation of mesothelioma cells. Then, we postulated that sphingosine might suppress proliferation of mesothelioma cells by inhibiting PKC-δ.

Mesothelioma cell proliferation is suppressed by inhibiting PKC-δ

To obtain evidence for sphingosine-induced PKC-δ inhibition, we carried out cell-free PKC assay. PKC-δ activity in the absence of sphingosine was 2.26 ± 0.32 pmol/min, but the activity was significantly attenuated in the presence of sphingosine (0.76 ± 0.06 pmol/min) (Fig. 6). This confirms that sphingosine inhibits PKC-δ.

For cells transfected with the PKC-δ siRNA, expression of PKC-δ protein was clearly reduced than the expression for cells transfected with NC siRNA (Fig. 7A, B, C, D), confirming PKC-δ knock-down. Spontaneous mesothelioma cell growth was significantly inhibited

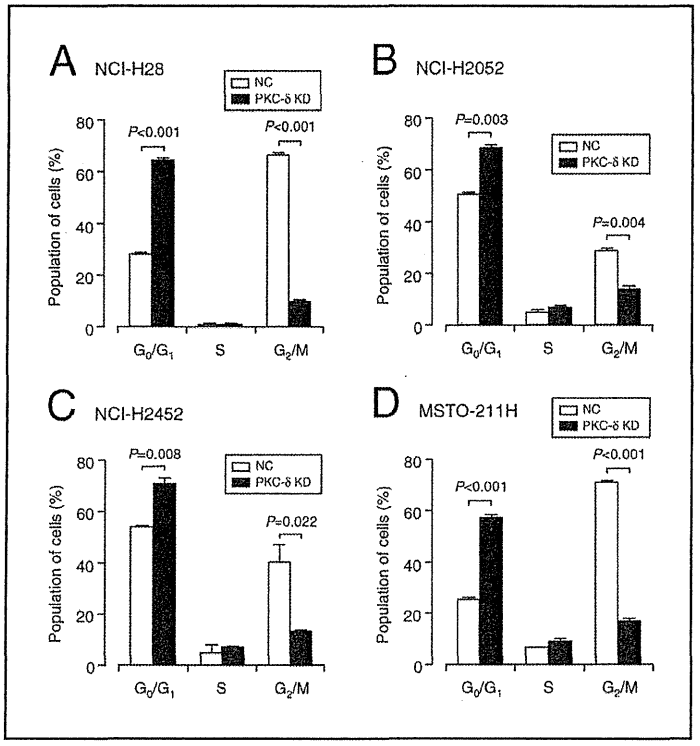

Fig. 8. The effect of PKC-δ knock-down on cell cycling. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were transfected with the NC siRNA (NC) or the PKC-δ siRNA (PKC-δ KD), and 72 h later cell cycle analysis was carried out. In the graphs, each column represents the mean (± SEM) percentage for phases of cell cycling (n=4 independent experiments). *P* values, Dunnett's test.

by knocking-down PKC-δ, with the order of the potential: NCI-H2052 cells=MSTO-211H cells>NCI-H2452 cells>>NCI-H28 cells (Fig. 7A, B, C, D). This indicates that PKC-δ promotes mesothelioma cell proliferation.

Sphingosine suppresses mesothelioma cell proliferation by inducing cell cycle arrest at the G_0/G_1 phase

In the cell cycle analysis, knocking-down PKC-δ significantly increased the population of cells at the G_0/G_1 phase of cell cycling, but it otherwise decreased the population at the G_2/M phase for all the cell types (Fig. 8). This implies that PKC-δ accelerates cell cycling for mesothelioma cells, i.e., inhibiting PKC-δ causes cell cycle arrest at the G_0/G_1 phase.

Discussion

The results of the present study demonstrate that sphingosine suppresses mesothelioma cell proliferation. Sphingosine is recognized to bind to and activate SDK, that is produced through proteolytic processing of PKC-δ [2]. Sphingosine induces apoptosis in hippocampal neurons and astrocytes by activating SDK, to phosphorylate 14-3-3 protein, thereby dissociating Bax and disrupting mitochondrial membrane potentials, and then leading to activation of caspase-9 and the effector caspase-3 [1]. In the present study, sphingosine did not increase SDK production, which would exclude the possibility for the implication of SDK in sphingosine-induced suppression of mesothelioma cell proliferation.

Of PKC isozymes cloned PKC-δ as well as the other novel PKCs including PKC-ε, -η, -θ, and -μ, is activated by signaling cascades linked to phospholipase A_2 in a Ca^{2+}-independent manner [7, 8]. PKC-δ is activated by translocation towards the membrane surface [9]. Sphingosine inhibits PKC activation induced by diacylglycerol, phorbol dibutyrate, calcium, unsaturated fatty acids or other lipids [10, 11]. PKC binds to membranes through interactions with diacylglycerol and negatively charged phosphatidylserine. Sphingosine may be localized

in regions of acidic lipids, thereby inhibiting PKC binding to membranes and activity [12-15]. Sphingosine, alternatively, inhibits the enzyme phosphatidic acid phosphohydrolase, that generates diacylglycerol production from phosphatidic acid produced by phospholipase D-catalyzed hydrolysis of phosphatidylcholine [16-18]. In the cell-free PKC assay, sphingosine attenuated PKC-δ activity. This indicates that sphingosine inhibits PKC-δ through its direct binding. Notably, rottlerin, an inhibitor of PKC-δ, further enhanced sphingosine-induced suppression of mesothelioma cell growth or rottlerin alone without sphingosine inhibited mesothelioma cell growth. Moreover, knocking-down PKC-δ significantly suppressed mesothelioma cell growth. It is indicated from these results that sphingosine suppresses mesothelioma cell proliferation by inhibiting PKC-δ. In the cell cycle analysis, knocking-down PKC-δ induced cell cycle arrest at the $G_0/_1$ phase for all the cell types used here. Overall, these results lead to a conclusion that sphingosine suppresses mesothelioma cell proliferation by inhibiting PKC-δ, to induce cell cycle arrest at the $G_0/_1$ phase.

Lines of evidence have pointed to the contrasting roles of PKC-δ in cell survival and cell death [19]. The pro- and anti-apoptotic function of PKC-δ not only depends on the cell type but also on the stimulus. PKC-δ promotes survival of a variety of cancer cells such as non–small cell lung cancer, breast cancer, pancreatic cancer, liver cancer, and chronic lymphocytic leukemia cells. PKC-δ protects glioma cells from the apoptosis induced by TRAIL, and phosphorylation of PKC-δ on Tyr155 and its cleavage are essential for the anti-apoptotic effect of PKC-δ [20]. Phosphorylation of PKC-δ at Tyr332 is also required for protection against TRAIL-induced apoptosis [21]. Overexpression of PKC-δ increases cell proliferation, anchorage-independent growth, and resistance to apoptotic stimuli by elevating cyclin D1 level and hyperphosphorylating Rb in murine mammary NMuMG cells [22]. Conversely, PKC-δ antisense oligonucleotide and dominant-negative PKC-δ decrease survival of breast cancer MCF-7 and MDA-MB-231 cells [23]. As found with the present study, rottlerin enhances apoptosis in non–small cell lung cancer cells [24]. Rottlerin blocks DNA damage–induced apoptosis, but otherwise it potentiates receptor-induced apoptosis [25].

PKC-δ promotes cell survival via several well-known pro-survival pathways, that include NF-κB, Akt, and ERK. PKC-δ prevents apoptosis in colon cancer cells by inducing inhibitor of apoptosis protein-2 and FLICE-like inhibitory protein via NF-κB [26, 27]. PKC-δ suppresses autophagy in breast cancer cells via NF-κB [28]. Tumor necrosis factor (TNF) is recognized to induce translocation of PKC-δ to the nucleus, where it bound to the NF-κB RelA subunit and induced transactivation of p65/RelA [29]. PKC-δ protects MCF-7 breast cancer cells against TNF-related apoptosis-inducing ligand-mediated apoptosis [30]. Phosphoinositide-dependent kinase-1 may also participate in PKC-δ survival signaling in cells containing an activated p21Ras protein [31]. Activation of Akt and ERK, alternatively, is responsible for PKC-δ-mediated increase in anchorage-independent growth and resistance of pancreatic ductal cancer cells to apoptotic stimuli [32]. PKC-δ-induced cell proliferation in murine mammary cells is associated with activation of ERK/MAPK [22]. In contrast, PKC-δ-induced suppression of ERK1/2 is associated with the survival of MDA-MB-231 cells [33]. PKC-δ attenuates apoptosis by inducing phosphorylation and proteasomal degradation of the proapoptotic protein Bim via the MEK/MAPK pathway in immortalized and malignant keratinocytes [34]. Syk, a tyrosine kinase, promotes survival of B-cell chronic lymphocytic leukemia cells by stabilizing Mcl-1 in a PKC-δ-dependent manner [35]. PKC-δ activated by Syk phosphorylates and inhibits glycogen synthase kinase-3, causing stabilization of Mcl-1 and inhibition of apoptosis. It is presently unknown what signaling pathways underlie sphingosine/PKC-δ-dependent inhibition of proliferation in mesothelioma cells. To address this question, we are carrying out further experiments.

In conclusion, the results of the present study show that sphingosine inhibits PKC-δ, thereby inducing cell cycle arrest at the $G_0/_1$ phase, and then leading to suppression of mesothelioma cell proliferation. This may represent further insight into the sphingosine signaling pathway relevant to cell proliferation and death.

References

1 Kanno T, Nishizaki T: Sphingosine induces apoptosis in hippocampal neurons and astrocytes by activating caspase-3/-9 via a mitochondrial pathway linked to SDK/14-3-3 protein/Bax/cytochrome c. J Cell Physiol 2011;226:2329-2337.
2 Suzuki E, Handa K, Toledo MS, Hakomori S: Sphingosine-dependent apoptosis: a unified concept based on multiple mechanisms operating in concert. Proc Natl Acad Sci USA 2004;101:14788–14793.
3 Hamaguchi A, Suzuki E, Murayama K, Fujimura T, Hikita T, Iwabuchi K, Handa K, Withers DA, Masters SC, Fu H, Hakomori S: A sphingosine-dependent protein kinase that specifically phosphorylates 14-3-3 (SDK1) is identified as the kinase domain of PKCδ: a preliminary note. Biochem Biophys Res Commun 2003;307:589-594.
4 Hamaguchi A, Suzuki E, Murayama K, Fujimura T, Hikita T, Iwabuchi K, Handa K, Withers DA, Masters SC, Fu H, Hakomori S: Sphingosine-dependent protein kinase-1, directed to 14-3-3, is identified as the kinase domain of protein kinase Cδ. J Biol Chem 2003;278:41557-41565.
5 Phillips DC, Martin S, Doyle BT, Houghton JA: Sphingosine-induced apoptosis in rhabdomyosarcoma cell lines is dependent on pre-mitochondrial Bax activation and post-mitochondrial caspases. Cancer Res 2007;67:756-764.
6 Shimizu T, Kanno T, Tanaka A, Nishizaki T: α,β-DCP-LA selectively activates PKC-ε and stimulates neurotransmitter release with the highest potency among 4 diastereomers. Cell Physiol Biochem 2011;27:149-158.
7 Nishizuka Y: Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 1992;258:607-614.
8 Nishizuka Y: Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 1995;9:484-496.
9 Steinberg SF: Distinctive activation mechanisms and functions for protein kinase Cδ. Biochem J 2004;384:449–459.
10 Hannun YA, Loomis CR, Merrill AH Jr, Bell RM: Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets. J Biol Chem 1986;261:12604-12609.
11 el Touny S, Khan W, Hannun Y: Regulation of platelet protein kinase C by oleic acid. Kinetic analysis of allosteric regulation and effects on autophosphorylation, phorbol ester binding, and susceptibility to inhibition. J Biol Chem 1990;265:16437-16443.
12 Merrill AH Jr, Sereni AM, Stevens VL, Hannun YA, Bell RM, Kinkade JM Jr: Inhibition of phorbol ester-dependent differentiation of human promyelocytic leukemic (HL-60) cells by sphinganine and other long-chain bases. J Biol Chem 1986;261:12610-12615.
13 Merrill AH Jr, Nimkar S, Menaldino D, Hannun YA, Loomis C, Bell RM, Tyagi SR, Lambeth JD, Stevens VL, Hunter R, Liotta DC: Structural requirements for long-chain (sphingoid) base inhibition of protein kinase C in vitro and for the cellular effects of these compounds. Biochemistry 1989;28:3138-3145.
14 Bazzi MD, Nelsestuen GL: Mechanism of protein kinase C inhibition by sphingosine. Biochem Biophys Res Commun 1987;146:203-207.
15 Smith ER, Jones PL, Boss JM, Merrill AH Jr: Changing J774A.1 cells to new medium perturbs multiple signaling pathways, including the modulation of protein kinase C by endogenous sphingoid bases. J Biol Chem 1997;272:5640–5646.
16 Nakamura T, Abe A, Balazovich KJ, Wu D, Suchard SJ, Boxer LA, Shayman JA: Ceramide regulates oxidant release in adherent human neutrophils. J Biol Chem 1994;269:18384-18389.
17 Merrill AH Jr, Hannun YA, Bell RM: Introduction: Sphingolipids and their metabolites in cell regulation. Adv Lipid Res 1993;25:1-24.
18 Raeder EM, Mansfield PJ, Hinkovska-Galcheva V, Kjeldsen L, Shayman JA, Boxer LA: Sphingosine blocks human polymorphonuclear leukocyte phagocytosis through inhibition of mitogen-activated protein kinase activation. Blood 1999;93:686-693.
19 Basu A, Pal D: Two faces of protein kinase Cδ: the contrasting roles of PKCδ in cell survival and cell death. ScientificWorldJournal 2010;10:2272-2284.
20 Okhrimenko H, Lu W, Xiang C, Ju D, Blumberg PM, Gomel R, Kazimirsky G, Brodie C: Roles of tyrosine phosphorylation and cleavage of protein kinase Cδ in its protective effect against tumor necrosis factor-related apoptosis inducing ligand-induced apoptosis. J Biol Chem 2005;280:23643-23652.

21 Lu W, Lee HK, Xiang C, Finniss S, Brodie C: The phosphorylation of tyrosine 332 is necessary for the caspase 3-dependent cleavage of PKCδ and the regulation of cell apoptosis. Cell Signal 2007;19:2165–2173.
22 Grossoni VC, Falbo KB, Kazanietz MG, de Kier Joffe ED, Urtreger AJ: Protein kinase Cδ enhances proliferation and survival of murine mammary cells. Mol Carcinog 2007;46:381–390.
23 McCracken MA, Miraglia LJ, McKay RA, Strobl JS: Protein kinase Cδ is a prosurvival factor in human breast tumor cell lines. Mol Cancer Ther 2003;2:273–281.
24 Clark AS,West KA, Blumberg PM, Dennis PA: Altered protein kinase C (PKC) isoforms in non-small cell lung cancer cells: PKCδ promotes cellular survival and chemotherapeutic resistance. Cancer Res 2003;63:780–786.
25 Basu A, Miura A: Differential regulation of extrinsic and intrinsic cell death pathways by protein kinase C. Int J Mol Med 2002;10:541–545.
26 Wang Q, Wang X, Evers BM: Induction of cIAP-2 in human colon cancer cells through PKC delta/NF-κB. J Biol Chem 2003;278:51091–51099.
27 Wang Q, Wang X, Zhou Y, Evers BM: PKCδ-mediated regulation of FLIP expression in human colon cancer cells. Int J Cancer 2006;118:326–334
28 Mann AP, Verma A, Sethi G, Manavathi B, Wang H, Fok JY, Kunnumakkara AB, Kumar R, Aggarwal BB, Mehta K: Overexpression of tissue transglutaminase leads to constitutive activation of nuclear factor-κB in cancer cells: delineation of a novel pathway. Cancer Res 2006;66:8788–8795.
29 Lu ZG, Liu H, Yamaguchi T, Miki Y, Yoshida K: Protein kinase Cδ activates RelA/p65 and nuclear factor-κB signaling in response to tumor necrosis factor-α. Cancer Res 2009;69:5927–5935.
30 Zhang J, Liu N, Liu S, Liu Y, Zheng D: PKCδ protects human breast tumor MCF-7 cells against tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis. J Cell Biochem 2005;96:522–532.
31 Xia S, Chen Z, Forman LW, Faller DV: PKCδ survival signaling in cells containing an activated p21Ras protein requires PDK1. Cell Signal 2009;21:502–508.
32 Mauro LV, Grossoni VC, Urtreger AJ, Yang C, Colombo LL, Morandi A, Pallotta MG, Kazanietz MG, Bal de Kier Joffe ED, Puricelli LL: PKC Delta (PKCδ) promotes tumoral progression of human ductal pancreatic cancer. Pancreas 2010;39: e31–41.
33 Lonne GK, Masoumi KC, Lennartsson J, Larsson C: Protein kinase Cδ supports survival of MDA-MB-231 breast cancer cells by suppressing the ERK1/2 pathway. J Biol Chem 2009;284:33456–33465.
34 Quadros MR, Connelly S, Kari C, Abrams MT, Wickstrom E, Rodeck U: EGFR-dependent downregulation of Bim in epithelial cells requires MAPK and PKC-δ activities. Cancer Biol Ther 2006;5:498–504.
35 Baudot AD, Jeandel PY, Mouska X, Maurer U, Tartare-Deckert S, Raynaud SD, Cassuto JP, Ticchioni M, Deckert M: The tyrosine kinase Syk regulates the survival of chronic lymphocytic leukemia B cells through PKCδ and proteasome-dependent regulation of Mcl-1 expression. Oncogene 2009;28:3261–3273.

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INTRODUCTION TO REVIEW ARTICLES

Early mesothelioma revisited

Seiki Hasegawa

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Prognosis of malignant pleural mesothelioma (MPM) remains poor

Very recently, a randomized controlled trial successfully proved a significant reduction in lung cancer mortality with CT screening [1]. This was an epoch-making report. Although intuition had been telling us that early detection of lung cancer converts directly into reduced mortality, most previous studies have only proven an improved detection rate; they have failed to show any reduction of mortality from lung cancer [2–4]. The success of the present study was obtained by focusing on a high-risk population.

Although primary lung cancer had been—as MPM still is—considered a highly incurable disease, its mortality is currently much improved. For example, in the Japanese Lung Cancer Registry, the 5-year survival rate for all surgical cases was 51.9% in 1996 and 69.6% in 2004 [5]. This striking improvement in survival may be multifactorial, but early diagnosis and early treatment are undoubtedly the most important factors. In this registry, 48.1% of the 11663 surgical cases in 2004 were at pathological stage Ia, and the 5-year survival rate of this subgroup reached 85.1% [5].

On the other hand, MPM remains one of the most incurable malignancies, and its median survival time (MST) after diagnosis is approximately 1 year [6–8].

Therapy for MPM (just like other malignancies) has evolved considerably, and is still evolving: adjuvant chemotherapy [9], neoadjuvant chemotherapy [10], the introduction of pemetrexed [11–13], the reduction in mortality/morbidity after extrapleural pneumonectomy (EPP) [14, 15], the introduction of radical pleurectomy/decortication (P/D) [16–19], adjuvant radiotherapy [20, 21], the introduction of intensity-modulated radiotherapy (IMRT) [22–25], and so on. Trimodality treatment (TMT) with induction chemotherapy followed by extrapleural pneumonectomy (EPP) and postoperative radiation was introduced as an integration of the above developments, and is currently considered the most powerful therapeutic option [10, 26–32]. TMT is only feasible for highly selected patients with early MPM and excellent cardiopulmonary reserve. However, notwithstanding its high cost, its extremely high risk, and its severe deterioration of cardiopulmonary function, MST after TMT still does not reach 20 months in most large studies [29–31, 33], so some researchers are skeptical about aggressive surgery in MPM patients [33, 34].

In the above context, the current therapeutic options are not sufficient to provide acceptable survival in cases with non-early MPM.

Early treatment is essential for improving MPM prognosis

As was the case with lung cancer a few decades ago, there is currently no established evidence that the early discovery of MPM will enable the patient to be cured, or even that it will improve their survival by many months [35].

Lots of reports with low levels of evidence indicate that early-stage MPM provides better survival. Boutin [36] performed thoracoscopy in 188 MPM patients and found only nonspecific inflammation in 12 (6.5%). MST in these 12 patients was 28.3 months. with no treatment administered other than talc poudrage [37]. In the report from the Memorial Sloan–Kettering Cancer Center, MST after EPP or P/D (pleurectomy/decortication) in AJCC stage I

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patients was 38 months [38]. A Toronto group reported that the MST for the N0–1 subgroup that completed TMT was 59 months [31]. A benefit of early chemotherapy has also been demonstrated by a prospective randomized trial [39].

The above facts indicate that a better prognosis for MPM could be obtained if early detection and early treatment were to be achieved [40].

In this context, a practical way of obtaining better survival with the currently available therapeutic options may be to diagnose MPM earlier.

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References

1. Aberle DR, Adams AM, Berg CD et al (2011) Reduced lung-cancer mortality with low-dose computed tomographic screening. N Engl J Med 365(5):395–409. doi:10.1056/NEJMoa1102873
2. Infante M, Cavuto S, Lutman FR et al (2009) A randomized study of lung cancer screening with spiral computed tomography: three-year results from the DANTE trial. Am J Respir Crit Care Med 180(5):445–453. doi:10.1164/rccm.200901-0076OC
3. Dutt N, Hari DT (2011) CT screening for lung cancer: so near, yet so far. Thorax. doi:10.1136/thoraxjnl-2011-200762
4. Oken MM, Hocking WG, Kvale PA et al (2011) Screening by chest radiograph and lung cancer mortality: the Prostate, Lung, Colorectal, and Ovarian (PLCO) randomized trial. JAMA 306(17):1865–1873. doi:10.1001/jama.2011.1591
5. Sawabata N, Miyaoka E, Asamura H et al (2011) Japanese Lung Cancer Registry study of 11,663 surgical cases in 2004: demographic and prognosis changes over decade. J Thorac Oncol 6(7):1229–1235. doi:10.1097/JTO.0b013e318219aae2
6. Robinson BW, Lake RA (2005) Advances in malignant mesothelioma. N Engl J Med 353(15):1591–1603. doi:10.1056/NEJMra050152
7. Tsao AS, Wistuba I, Roth JA et al (2009) Malignant pleural mesothelioma. J Clin Oncol 27(12):2081–2090. doi:10.1200/JCO.2008.19.8523
8. Milano MT, Zhang H (2010) Malignant pleural mesothelioma: a population-based study of survival. J Thorac Oncol 5(11):1841–1848. doi:10.1097/JTO.0b013e3181f1cf2b
9. Sugarbaker DJ, Flores RM, Jaklitsch MT et al (1999) Resection margins, extrapleural nodal status, and cell type determine postoperative long-term survival in trimodality therapy of malignant pleural mesothelioma: results in 183 patients. J Thorac Cardiovasc Surg 117(1):54–63 (discussion 63–65)
10. Weder W, Kestenholz P, Taverna C et al (2004) Neoadjuvant chemotherapy followed by extrapleural pneumonectomy in malignant pleural mesothelioma. J Clin Oncol 22(17):3451–3457. doi:10.1200/JCO.2004.10.071
11. Scagliotti GV, Shin DM, Kindler HL et al (2003) Phase II study of pemetrexed with and without folic acid and vitamin B_{12} as front-line therapy in malignant pleural mesothelioma. J Clin Oncol 21(8):1556–1561. doi:10.1200/JCO.2003.06.122
12. Vogelzang NJ, Rusthoven JJ, Symanowski J et al (2003) Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. J Clin Oncol 21(14):2636–2644. doi:10.1200/JCO.2003.11.136
13. Rusch VW (2003) Pemetrexed and cisplatin for malignant pleural mesothelioma: a new standard of care? J Clin Oncol 21(14):2629–2630. doi:10.1200/JCO.2003.02.043
14. Sugarbaker DJ, Jaklitsch MT, Bueno R et al (2004) Prevention, early detection, and management of complications after 328 consecutive extrapleural pneumonectomies. J Thorac Cardiovasc Surg 128(1):138–146. doi:10.1016/j.jtcvs.2004.02.021
15. de Perrot M, McRae K, Anraku M et al (2008) Risk factors for major complications after extrapleural pneumonectomy for malignant pleural mesothelioma. Ann Thorac Surg 85(4):1206–1210. doi:10.1016/j.athoracsur.2007.11.065
16. Nakas A, Trousse DS, Martin-Ucar AE et al (2008) Open lung-sparing surgery for malignant pleural mesothelioma: the benefits of a radical approach within multimodality therapy. Eur J Cardiothorac Surg 34(4):886–891. doi:10.1016/j.ejcts.2008.06.010
17. Bolukbas S, Manegold C, Eberlein M et al (2011) Survival after trimodality therapy for malignant pleural mesothelioma: radical pleurectomy, chemotherapy with cisplatin/pemetrexed and radiotherapy. Lung Cancer 71(1):75–81. doi:10.1016/j.lungcan.2009.08.019
18. Shahin Y, Wellham J, Jappie R et al (2011) How successful is lung-preserving radical surgery in the mesothelioma and radical surgery-trial environment? A case-controlled analysis. Eur J Cardiothorac Surg 39(3):360–363. doi:10.1016/j.ejcts.2010.06.006
19. Teh E, Fiorentino F, Tan C et al (2011) A systematic review of lung-sparing extirpative surgery for pleural mesothelioma. J R Soc Med 104(2):69–80. doi:10.1258/jrsm.2010.100345
20. Rusch VW, Rosenzweig K, Venkatraman E et al (2001) A phase II trial of surgical resection and adjuvant high-dose hemithoracic radiation for malignant pleural mesothelioma. J Thorac Cardiovasc Surg 122(4):788–795. doi:10.1067/mtc.2001.116560
21. de Perrot M, Uy K, Anraku M et al (2007) Impact of lymph node metastasis on outcome after extrapleural pneumonectomy for malignant pleural mesothelioma. J Thorac Cardiovasc Surg 133(1):111–116. doi:10.1016/j.jtcvs.2006.06.044
22. Ahamad A, Stevens CW, Smythe WR et al (2003) Intensity-modulated radiation therapy: a novel approach to the management of malignant pleural mesothelioma. Int J Radiat Oncol Biol Phys 55(3):768–775
23. Allen AM, Czerminska M, Janne PA et al (2006) Fatal pneumonitis associated with intensity-modulated radiation therapy for mesothelioma. Int J Radiat Oncol Biol Phys 65(3):640–645. doi:10.1016/j.ijrobp.2006.03.012
24. Rice DC, Stevens CW, Correa AM et al (2007) Outcomes after extrapleural pneumonectomy and intensity-modulated radiation therapy for malignant pleural mesothelioma. Ann Thorac Surg 84(5):1685–1692 (discussion 1692–1683). doi:10.1016/j.athoracsur.2007.04.076
25. Chi A, Liao Z, Nguyen NP et al (2011) Intensity-modulated radiotherapy after extrapleural pneumonectomy in the combined-modality treatment of malignant pleural mesothelioma. J Thorac Oncol 6(6):1132–1141. doi:10.1097/JTO.0b013e3182199819
26. Neragi-Miandoab S (2006) Multimodality approach in management of malignant pleural mesothelioma. Eur J Cardiothorac Surg 29(1):14–19. doi:10.1016/j.ejcts.2005.10.008
27. Rea F, Marulli G, Bortolotti L et al (2007) Induction chemotherapy, extrapleural pneumonectomy (EPP) and adjuvant hemithoracic radiation in malignant pleural mesothelioma (MPM): feasibility and results. Lung cancer 57(1):89–95. doi:10.1016/j.lungcan.2007.02.004
28. Weder W, Stahel RA, Bernhard J et al (2007) Multicenter trial of neo-adjuvant chemotherapy followed by extrapleural pneumonectomy in malignant pleural mesothelioma. Ann Oncol 18(7):1196–1202. doi:10.1093/annonc/mdm093

29. Krug LM, Pass HI, Rusch VW et al (2009) Multicenter phase II trial of neoadjuvant pemetrexed plus cisplatin followed by extrapleural pneumonectomy and radiation for malignant pleural mesothelioma. J Clin Oncol 27(18):3007–3013. doi:10.1200/JCO.2008.20.3943
30. Van Schil PE, Baas P, Gaafar R et al (2010) Trimodality therapy for malignant pleural mesothelioma: results from an EORTC phase II multicentre trial. Eur Respir J 36(6):1362–1369. doi:10.1183/09031936.00039510
31. de Perrot M, Feld R, Cho BC et al (2009) Trimodality therapy with induction chemotherapy followed by extrapleural pneumonectomy and adjuvant high-dose hemithoracic radiation for malignant pleural mesothelioma. J Clin Oncol 27(9):1413–1418. doi:10.1200/JCO.2008.17.5604
32. Yamanaka T, Tanaka F, Hasegawa S et al (2009) A feasibility study of induction pemetrexed plus cisplatin followed by extrapleural pneumonectomy and postoperative hemithoracic radiation for malignant pleural mesothelioma. Jpn J Clin Oncol 39(3):186–188. doi:10.1093/jjco/hyn145
33. Trousse DS, Avaro JP, D'Journo XB et al (2009) Is malignant pleural mesothelioma a surgical disease? A review of 83 consecutive extra-pleural pneumonectomies. Eur J Cardiothorac Surg 36(4):759–763. doi:10.1016/j.ejcts.2009.04.044
34. Treasure T, Lang-Lazdunski L, Waller D et al (2011) Extrapleural pneumonectomy versus no extra-pleural pneumonectomy for patients with malignant pleural mesothelioma: clinical outcomes of the Mesothelioma and Radical Surgery (MARS) randomised feasibility study. Lancet Oncol 12(8):763–772. doi:10.1016/S1470-2045(11)70149-8
35. Scherpereel A, Astoul P, Baas P et al (2010) Guidelines of the European Respiratory Society and the European Society of Thoracic Surgeons for the management of malignant pleural mesothelioma. Eur Respir J 35(3):479–495. doi:10.1183/09031936.00063109
36. Boutin C, Rey F (1993) Thoracoscopy in pleural malignant mesothelioma: a prospective study of 188 consecutive patients. Part 1: diagnosis. Cancer 72(2):389–393
37. Boutin C, Rey F, Gouvernet J et al (1993) Thoracoscopy in pleural malignant mesothelioma: a prospective study of 188 consecutive patients. Part 2: prognosis and staging. Cancer 72(2):394–404
38. Flores RM, Pass HI, Seshan VE et al (2008) Extrapleural pneumonectomy versus pleurectomy/decortication in the surgical management of malignant pleural mesothelioma: results in 663 patients. J Thorac Cardiovasc Surg 135(3):620–626, 626.e1–626.e3. doi:10.1016/j.jtcvs.2007.10.054
39. O'Brien ME, Watkins D, Ryan C et al (2006) A randomised trial in malignant mesothelioma (M) of early (E) versus delayed (D) chemotherapy in symptomatically stable patients: the MED trial. Ann Oncol 17(2):270–275. doi:10.1093/annonc/mdj073
40. Pass HI, Carbone M (2009) Current status of screening for malignant pleural mesothelioma. Semin Thorac Cardiovasc Surg 21(2):97–104. doi:10.1053/j.semtcvs.2009.06.007