Abstract. Background: We have previously found that Wnt signaling is activated in mesothelioma cells. To clarify the effect of blocking Wnt signaling in mesothelioma, the expression of dishevelled (Dvl), an intermediary of Wnt signaling, was down-regulated by a reformed type of small interfering RNA (siRNA), stealth RNAi, which can reduce the cytotoxic interferon response unlike conventional siRNA.

Materials and Methods: Mesothelioma cell lines were transfected with stealth RNAi of Dvl, and cell growth and colony formation were examined. The synergistic effect on cell growth of Dvl stealth RNAi and cisplatin in combination was evaluated. Results: Dvl stealth RNAi down-regulated the expression of Dvl-3 in mesothelioma cells and induced cell cycle aberration which caused suppression of cell growth. Colony formation was also suppressed. Dvl stealth RNAi and cisplatin in combination suppressed cell growth synergistically.

Conclusion: Our data suggest that inhibition of Wnt signaling leads to significant antitumor effects.

Malignant pleural mesothelioma is an asbestos-related malignancy characterized by rapidly progressive and diffuse local growth, late metastases and death. Approximately 1,000 people die of this disease in Japan annually. The incidence of this disease is rising worldwide (1, 2). The mechanism by which mesothelial cells undergo neoplastic transformation is largely unknown, although recent evidence suggests a multi-step process involving both activation of oncogenes and inactivation of tumor suppressor genes (3). Unfortunately, the results of standard therapies (surgery, chemotherapy and radiation) remain disappointing. Recently-developed anti-folate reagents are expected to prolong survival (4, 5); however, newer therapies based on an improved molecular understanding of mesothelioma are needed.

Aberrant activation of the Wnt signaling pathway is implicated in the development of a broad spectrum of tumors (6). Previously, we have found that Wnt signaling is activated in mesothelioma cells and that blockage of Wnt signaling with antibodies of Wnt-1 or -2, small interfering RNA (siRNA) of Wnt-1 or -2, or dominant-negative "dishevelled" (Dvl), suppressed growth or tumorigenesis of mesothelioma cells in athymic mouse (7-9).

To confirm the role of Dvl in mesothelioma cells, we prepared a re-formed type of small interfering RNA (siRNA), stealth RNAi of Dvl, and transfected it to mesothelioma cells. The effect on mesothelioma cell growth of the Dvl stealth RNAi alone and together with an anticancer drug, cisplatin, was evaluated.

Materials and Methods

Cell lines and cell culture. Mesothelioma cell lines were obtained as follows: REN was a generous gift from Dr. Steven Albelda (University of Pennsylvania, Philadelphia, PA, USA); NCI-H290 was from the National Institutes of Health (Frederick, MD, USA); NCI-428 was from the American Type Culture Collection (ATCC;
Manassas, VA, USA). All cell lines were cultured in RPMI-1640 complete media (ATCC) containing 10% fetal bovine serum (FBS) (ATCC) in a 75 cm² tissue culture flask in a 37°C 5% CO₂ incubator. Medium changes were performed every 3 days. When the cells reached 80% confluent, they were treated with 0.5% Trypsin / 0.2% ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich, St. Louis, USA) and then cells were passaged 1 in 4.

**Western blot and immunohistochemistry.** Cells were scraped and then washed with Tris-buffered saline (25 mM Tris-HCl pH 8.0, 150 mM NaCl), containing 0.1 mM phenylmethlysulfonylfluoride (PMSF), and centrifuged at 1,000 xg for 5 min. The cells were lysed with 9 volumes of lysing buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1 mM PMSF) and centrifuged at 10,000 xg for 5 min. The supernatant was mixed with an equal volume of 4% SDS containing 10% 2-mercaptoethanol. The whole cell lysate aliquots were separated on 4-15% gradient SDS-polyacrylamide gels, subjected to 10% SDS-Page and electrotransferred to Hybond-ECL membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for Western blotting. The blots were incubated with Dvl-3 (4D3) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or β-actin (Sigma Chemical Co., St. Louis, MO, USA). Antigen-antibody complexes were detected by an ECL blotting analysis system (Amersham Pharmacia Biotech).

**Transfection with siRNA of Dvl-3.** Dvl-3 siRNA (Stealth RNAi) was prepared by Invitrogen (Carlsbad, CA, USA). The sequence of the siRNA targeting Dvl-3 was GGGCCAGACCAAGAUCAUCUA CCACU. The control siRNA sequence used was GGCCAGAA CUAGUACAUCCCGAACU. Transfection was performed using Lipofectamine™ 2000 (Invitrogen) in accordance with the manufacturer’s instructions. Cells were seeded into 6-well plates at 1x10⁴ cells/well; 24 h later cells were transfected with 4 pmol of siRNA using 4 ìl of Lipofectamine™ 2000. The cells were incubated for 24 h at 37°C; washed once with phosphate-buffered saline (PBS), and then incubated using RPMI-1640 with 10% FBS.

**Cytotoxicity assays.** A modification of the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was applied for estimating cell viability using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), which contains 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) dye. In brief, cells were seeded at 5x10⁴ cells/well in 96-well plates 24 h after transfection with siRNA. Cell viability was estimated at 24, 48, 72 and 96 h after plating. WST-8 was added 2 h before the end of culture and the absorbance was measured at 450 nm using a Spectra Max 250 microtiter plate reader (Molecular Device, Sunnyvale, CA, USA). Experiments were performed at least 6 times. Furthermore, at 24 h after plating, the cells were exposed to 10 or 50 µM of cisplatin or dimethyl sulfoxide (DMSO) as control, and cell viability was estimated in the same manner.

**Cell cycle analysis.** After 48 h of RNAi transfection, cells were collected. The cells were applied to a CycleTest PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA, USA), according to the manufacturer’s instructions. Cell cycle analysis was determined on a flow cytometer (BD Biosciences).

**Statistical methods.** Results are expressed as means±standard deviation. All statistical comparisons were made with a two-sided Student’s t-test. A p-value of less than 0.05 was considered to be statistically significant.

**Results**

Stealth RNAi of Dvl-3 down-regulated expression of Dvl-3 in mesothelioma cells. Mesothelioma cell lines REN, H290 and H28 express Dvl-3. At 48 h after transfection with Dvl-3 stealth RNAi, expression of Dvl-3 was down-regulated in REN, H290 and H28 cell line (Figure 1).

**Down-regulation of Dvl-3 suppressed growth and colony formation of mesothelioma cells.** After 48 h of transfection with Dvl-3 stealth RNAi, the number of viable REN and H290 cells was statistically significantly reduced as
compared with those transfected with control siRNA. The number of viable H28 cells was reduced after 72 h of transfection (Figure 2).

Colony counts of REN, H290 and H28 cell lines were reduced by their transfection with Dvl-3 stealth RNAi, compared to those cells transfected with control siRNA (Figure 3).

Down-regulation of Dvl-3 induced G1 population increase. After 48 h of transfection with Dvl-3 stealth RNA, the population of REN, H290 and H28 cells in the G1 stage increased (Figure 4).

Down-regulation of Dvl-3 plus cisplatin suppressed growth of mesothelioma cells synergistically. The effect on cell growth of Dvl-3 stealth RNAi plus the anticancer drug cisplatin was examined. The growth of REN and H290 cells was synergistically suppressed with Dvl-3 stealth RNAi after 48 h of incubation with cisplatin (Figure 5). In H28 cells, the addition of cisplatin only slightly enhanced the inhibition due to transfection with Dvl-3 stealth RNAi.

**Discussion**

We have shown that down-regulation of Dvl-3 induced aberration of the cell cycle and suppressed cell growth in mesothelioma cells. Of particular importance is the fact that colony formation was effectively suppressed by stealth RNAi of Dvl-3, which supports our previous finding that $\triangle$PDZ-Dvl mutant suppressed the colony formation in soft agar, but not the cell growth (7). The Wnt signal might therefore be more profoundly related to tumor formation in mesothelioma.

Previously, we used siRNA to repress the expression of Dvl and showed that the growth of non-small cell lung cancer H1703 cells was suppressed (10); however, suppression of mesothelioma cell growth had not been
shown. In the present study, stealth RNAi suppressed the growth of mesothelioma REN, H290 and H28 cells. This suppression of the toxic interferon response due to conventional siRNA explains the basic function of Dvl.

Suppressed expression of Dvl-3 caused G1 arrest in mesothelioma REN, H290 and H28 cells, which suggests that Dvl-3 is related to cell cycle regulation. Previously, cyclin D1, a cell cycle regulator, was shown to be the downstream target of Wnt signaling (11). But in our experiment the expression of cyclin D1 was not affected by suppression of Dvl-3, so another pathway remains to be clarified.

Recently, the new anti-folate reagents pemetrexed and raltitrexed in combination with cisplatin have been shown to prolong survival for mesothelioma patients, compared to those treated with cisplatin alone (4, 5). In the hope that suppression of Dvl may lead to a new treatment of mesothelioma, combination with cisplatin was examined here. After application of Dvl-3 stealth RNAi, cisplatin was given to mesothelioma cells. Their growth was effectively inhibited by Dvl-3 stealth RNAi plus cisplatin. Experiments were performed a minimum of 6 times. *p<0.05 compared to control.

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References


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