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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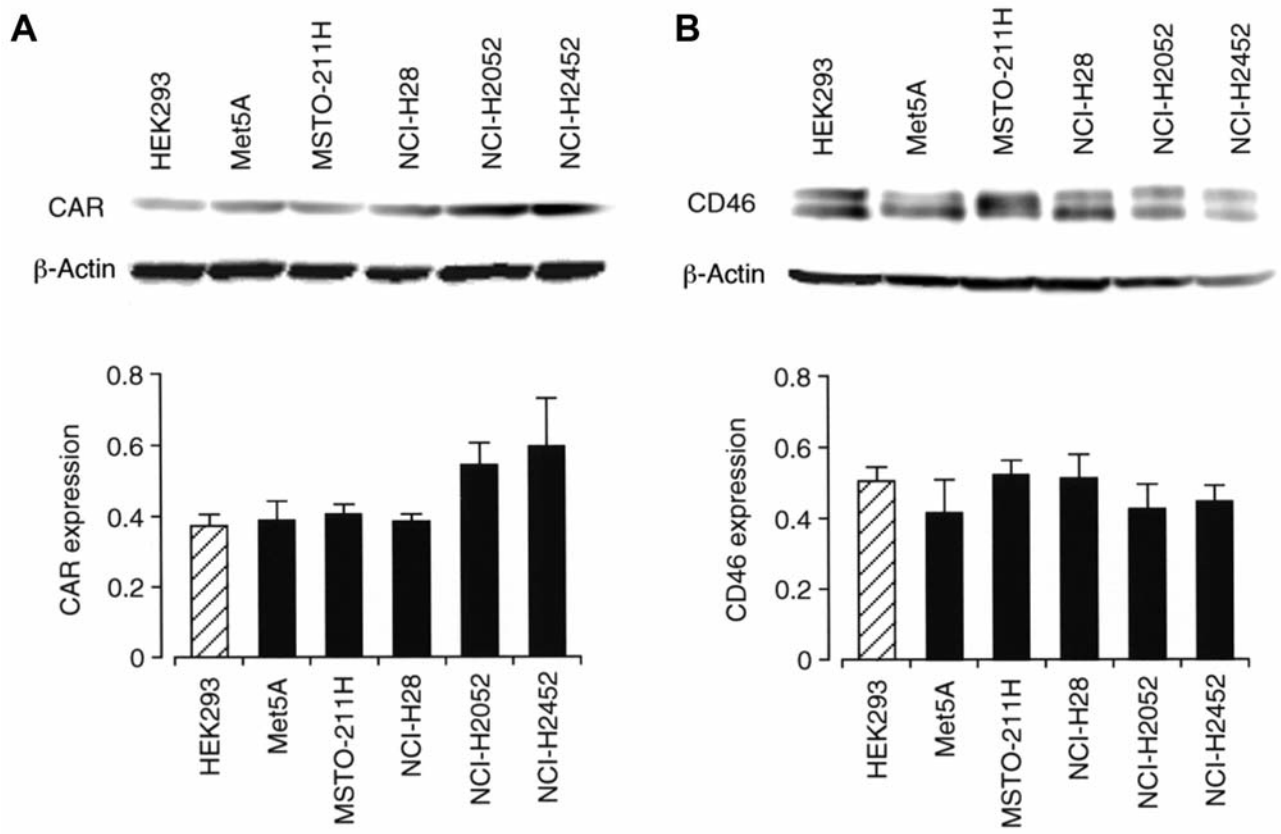

Figure 1. *Expression of CAR and CD46 protein. Western blotting for CAR (A) and CD46 (B) were carried out in cells, as indicated. The expression levels of each protein were normalized to those for β-actin. In the graphs, each column represents the mean (±SEM) expression of each protein (n=4 independent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

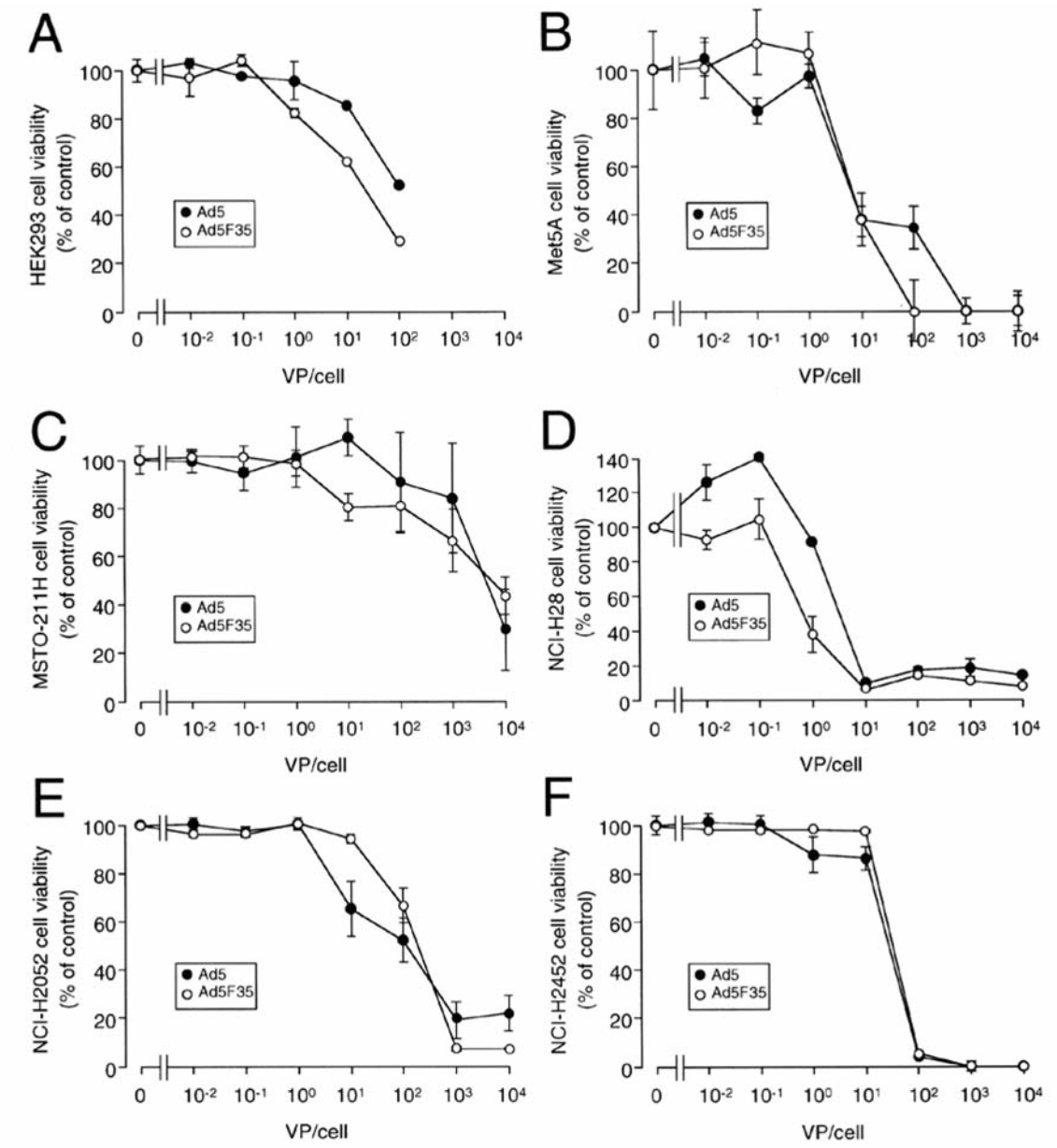

Figure 2. *Oncolytic effects of Ad5/MKp-E1 and Ad5F35/MKp-E1. Cell viability was assayed 5 days after infection using an Alamar Blue in HEK293 (A), Met5A (B), MSTO-211H (C), NCI-H28 (D), NCI-H2052 (E), and NCI-H2452 cells (F). In the graphs, each point represents the mean (±SEM) percentage of basal levels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

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