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Enhanced antitumor efficacy of fiber-modified, midkine promoter-regulated oncolytic adenovirus in human malignant mesothelioma

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Oncolytic virotherapy using adenoviruses has potential for therapeutic benefits in malignant mesothelioma. However, the downregulation of coxsackie virus/adenovirus receptor (CAR) expression is frequently a critical rate-limiting factor that impedes the effectiveness of adenovirus serotype 5 (Ad5)-based vectors in many cancer types. We evaluated CAR (Ad5 receptor) and CD46 (adenovirus serotype 35 [Ad35] receptor) expression in six human malignant mesothelioma cell lines. Very low CAR expression was observed in MSTO-211H and NCI-H2052 cells, whereas the other cell lines showed strong expression. In contrast, CD46 was highly expressed in all mesothelioma cell lines. On this basis, we replaced the CAR binding sequence of Ad5 with the CD46 binding sequence of Ad35 in the replication-defective adenoviruses and the tumor-specific midkine promoter-regulated oncolytic adenoviruses. By this fiber modification, the infectivity, virus progeny production, and *in vitro* cytocidal effects of the adenoviruses were significantly enhanced in low CAR-expressing MSTO-211H and NCI-H2052 cells, also resulting in similar or even higher levels in high CAR-expressing mesothelioma cell lines. In MSTO-211H xenograft models, the fiber-modified oncolytic adenovirus significantly enhanced antitumor effect compared to its equivalent Ad5-based vector. Our data demonstrate that Ad35 fiber modification of binding tropism in a midkine promoter-regulated oncolytic Ad5 vector confers transductional targeting to oncolytic adenoviruses, thereby facilitating more effective treatment of malignant mesothelioma. (*Cancer Sci* 2013; 104: 1433–1439)

Malignant mesothelioma, often caused by exposure to asbestos, is one of the most lethal cancers.[1,2] Conventional therapies for malignant mesothelioma include surgical resection, chemotherapy, and radiotherapy, but are generally non-curative.[2,3] Accordingly, new therapeutic options are urgently needed. Oncolytic virotherapy represents a highly promising approach to cancer treatment, and clinical trials of several replicating viruses and vector systems are ongoing.[4]

A crucial factor in determining the therapeutic window for oncolytic virotherapy is the tumor selectivity of virus replication, which can be enhanced by substituting constitutively active native viral promoters with cellular promoters from genes highly upregulated in cancer cells. Midkine (MDK) is a basic heparin-binding growth factor and a developmentally important retinoic acid-responsive protein strongly induced during midgestation.[5] MDK is implicated in cancer development because of its mitogenic effect,[6,7] in the promotion of angiogenesis,[8] and for its antiapoptotic,[9,10] fibrinolytic,[11] and transforming[12] activities. MDK expression in normal human adult tissues is limited.[5,13–15] In contrast, MDK is overexpressed in various human cancers,[13,14,16–20] compared to adjacent normal tissues. We previously showed[21] that MDK expression is also highly upregulated in malignant mesothelioma compared to that in normal primary mesothelial and smooth muscle cells.

Accordingly, we found that the tumor-specific MDK promoter conferred tumor-selective transcriptional targeting on oncolytic adenoviruses,[21] which proved highly effective in mesothelioma models. However, infection with conventional Ad5-based oncolytic vectors is limited by low physical binding efficiency because Ad5 uses CAR as its main cellular receptor[22–25] and, although CAR is ubiquitously expressed in normal epithelial cells, its expression is downregulated in many tumor types.[25,26] Moreover, previous studies[26–28] suggest that tumor progression and aggressiveness correlates with suppressed CAR expression, so this limitation is exacerbated in advanced cancers. Downregulation of CAR expression in tumors therefore represents a significant obstacle to the use of Ad5 vectors for oncolytic virotherapy. Hence, modification of the adenovirus capsid, particularly of the viral fiber knob protein, which mediates primary binding of the virus to its cellular target, has been attempted to facilitate CAR-independent binding.

The adenovirus family comprises over 50 human serotypes.[29] Among these, Ad35 uses the complement-regulating protein CD46 as its cellular receptor.[30] CD46 is expressed on all nucleated cells at a low level, but its expression is greatly upregulated in cancer cells, including breast,[31–33] esophageal,[34] colon,[31,35] liver,[33,36] pancreatic,[32] prostate,[33] and ovarian[35] cancer cells. We evaluated CD46 and CAR expression in multiple mesothelioma cell lines. Subsequently, we investigated whether the antitumor efficacy of MDK-regulated Ad5-based oncolytic vectors improved *in vitro* and *in vivo* following modification of binding tropism through the replacement of the cellular binding domain of the Ad5 fiber knob with that of Ad35. Our results indicated that the dual-targeted adenovirus, combining cancer cell-selective binding tropism and transcriptional regulation, represents an effective strategy for malignant mesothelioma treatment by oncolytic virotherapy.

Materials and Methods

Cell lines. Human adult normal mesothelial cells (NMC) were purchased from Zen-Bio (Research Triangle Park, NC, USA) and maintained in mesothelial cell growth medium (Zen-Bio). Immortalized human mesothelial Met5A and four human mesothelioma cell lines, MSTO-211H, NCI-H2052, NCI-H2452, and

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NCI-H28, were obtained from ATCC (Manassas, VA, USA). Additional human mesothelioma cells, ACC-MESO-1 and ACC-MESO-4, were purchased from the RIKEN BioResearch Center (Tsukuba, Ibaraki, Japan). These cells were grown in Roswell Park Memorial Institute 1640 medium (RPMI-1640; Nacalai Tesque, Kyoto, Japan) and supplemented with 10% FBS (HyClone, Logan, UT, USA). The human embryonic kidney (HEK) 293 cells (HEK293; Microbix, Toronto, ON, Canada)[37] were cultured in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque) supplemented with 10% FBS. Human dermal fibroblasts and their specific media were purchased from Cell Systems (Kirkland, WA, USA). All cells were grown in 5% CO_2 at 37°C.

Expression profile analysis of CAR and CD46. Cells were stained with mouse monoclonal anti-human antibodies for CAR (ab9891) and CD46 (ab789) (both Abcam, Cambridge, UK), then with an Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (A11001; Molecular Probes, Eugene, OR, USA), and analyzed on a FACSCalibur flow cytometer with CellQuest software version 3.1f (Becton Dickinson Japan, Tokyo, Japan).

Vector plasmids and virus production. The recombinant adenoviral vectors used in these experiments included an EGFP gene expression cassette and were constructed using the Adeno Expression System (Clontech, Mountain View, CA, USA). First, a marker gene cassette containing EGFP driven by the human CMV promoter[21] was subcloned into the pShuttle2 backbone (Clontech) to generate the intermediate construct, pShuttle2G. For replication-competent oncolytic adenoviruses, MDK-E1 cassettes[21] were inserted into pShuttle2G to generate pShuttle2G-MDK-E1. Similarly, for replication-defective adenoviruses, PGK promoter-driven βgal reporter gene cassette was inserted into pShuttle2G to generate pShuttle2G-PGK-βgal. To construct pAdeno5/F35, the CAR-binding region of the Ad5 fiber knob on the pAdeno-X vector (Clontech) was replaced with the CD46-binding region of the Ad35 fiber from the RHSP vector (Avior Therapeutics, Seattle, WA, USA). For final construction of the recombinant adenoviral vectors, pShuttle2G-PGK-βgal and pShuttle2G-MDK-E1 were subcloned into pAdeno-X and pAdeno5/F35 to generate pAd5-PGK-βgal (Ad843), pAd5/F35-PGK-βgal (Ad844), pAd5-MDK-E1 (Ad888), and pAd5/F35-MDK-E1 (Ad889) (Fig. 2).

All vectors were propagated in HEK293 cells, purified by CsCl ultracentrifugation, and dialyzed against 10 mM Tris–HCl buffer (pH 8.0) with 10% glycerol. The titers of the vectors were determined by conventional limiting dilution on HEK293 cells and shown to possess similar viral yield. The particle : pfu ratios of these vectors ranged from 16.3 to 25.1. Titers were also determined by EGFP expression using a FACSCalibur flow cytometer (Becton Dickinson Japan) as TU per milliliter.

Virus infectivity assay. To compare the infectivity of adenoviral vectors with Ad5 fiber and Ad5/F35-modified fiber, 1×10^5 cells/well were seeded in 24-well plates and infected with serially diluted Ad843 or Ad844. Twenty-four hours later, EGFP titers were determined by flow cytometry, compared between Ad843 and Ad844 (Ad844/Ad843), after normalizing to baseline values in NMC.

Viral progeny production assay. To assay the production of virus progeny, 5×10^5 cells/well were seeded in six-well plates and infected with adenoviruses at a multiplicity of infection (MOI) of 100 viral particles per cell in 2 mL of 5% FBS medium. After 3 h, the infected cells were washed three times with PBS and incubated in growth medium. Forty-eight hours after infection, cells and media were harvested, frozen/thawed three times, centrifuged, and serial dilutions of the virus supernatants were titered on HEK293 cells for EGFP expression using flow cytometry.

***In vitro* cytotoxicity assay.** To determine the oncolytic activity of each adenovirus in various cell lines, cells were cultured on 24-well plates (1×10^5 cells/well) and infected with adenoviruses at various MOIs. Half the supernatant medium was replaced with fresh medium containing 4% FBS every day. On day 8, cells were fixed with 10% buffered formalin containing 1% crystal violet for 30 min followed by three washes in tap water and air-drying.

For quantitative analysis of the cytocidal effect of each adenovirus, three sets of 96-well tissue culture plates containing 5×10^3 cells per well were infected with Ad888 or Ad889 at various MOIs. On days 2, 4, and 8 postinfection, viable cells of triplicate cultures were determined using the Alamar blue method according to the manufacturer's instructions (Alamar Biosciences, Sacramento, CA, USA). In brief, 3 h after Alamar blue was aseptically added to the cultures, fluorescence was measured using an ARVO X4 multilabel plate reader (Perkin-Elmer, Waltham, MA, USA) with a 544-nm excitation wavelength and a 590-nm emission wavelength. The percentage of viable cells was determined by calculating the fluorescence of viable cells compared to wells containing no viral vectors.

Subcutaneous human malignant mesothelioma xenograft models. All animal experiments were carried out according to institutional guidelines as per an approved protocol. Human mesothelioma xenografts were established in 6–7-week-old female BALB/c-nu/nu (nude) mice (Charles River Japan, Yokohama, Japan) by s.c. inoculation of 1×10^6 MSTO-211H cells into the right dorsal flank. When tumors reached a diameter of approximately 5 mm, mice were intratumorally injected with 100 μL PBS, Ad844, Ad888, or Ad889 (5×10^7 TU) on days 0 and 4 ($n = 11$/group). The mice were observed closely, and tumors were measured twice a week. Tumor volume was calculated as: $a \times b^2 \times 0.5$, where a and b represent the large and small diameters, respectively.

Statistical analysis. Results are presented as the means ± SD. The statistical significance of differences was calculated using Student's t-test, and a P-value of <0.05 was considered significant.

Results

Cell surface expression of CAR and CD46 in human mesothelioma cells. Both CAR and CD46 expression was evaluated by flow cytometry (Fig. 1) in Met5A immortalized human mesothelial cells and six human mesothelioma cell lines. The CAR expression was very low in MSTO-211H and NCI-H2052 cells, whereas the other cell lines showed higher levels of expression. These findings coincided with our previous observation[21] that NCI-H2052 is resistant to both CMV- and MDK-regulated oncolytic Ad5 vectors, suggesting that CAR binding is a rate-limiting step for transduction in this cell line. In contrast, CD46 was highly expressed in all mesothelioma cell lines. Previously,[21] we found that the MDK promoter is also highly active in these mesothelioma cells compared to Met5A cells. Accordingly, these findings suggest that the use of Ad35 fiber to modify binding tropism in MDK-regulated oncolytic Ad5 vectors confers more effective transduction in all these mesothelioma cell lines.

Design and production of oncolytic and control adenoviruses. As shown in Figure 2, four different adenoviruses were constructed and tested. Ad888 is an oncolytic adenovirus with a wild-type Ad5 fiber, in which the expression of an adenoviral E1 gene is regulated by the MDK promoter. Ad889 is the equivalent MDK promoter-regulated vector with the Ad5/F35-modified fiber. As controls, Ad843 and Ad844 are replication-defective adenoviruses with either Ad5 fiber or Ad5/F35-modified fiber, respectively, and contain an MDK promoter-driven βgal marker gene instead of the MDK-E1 expression cassette in order to adjust the genome size of four adenoviruses to be the same. All vectors have an independent

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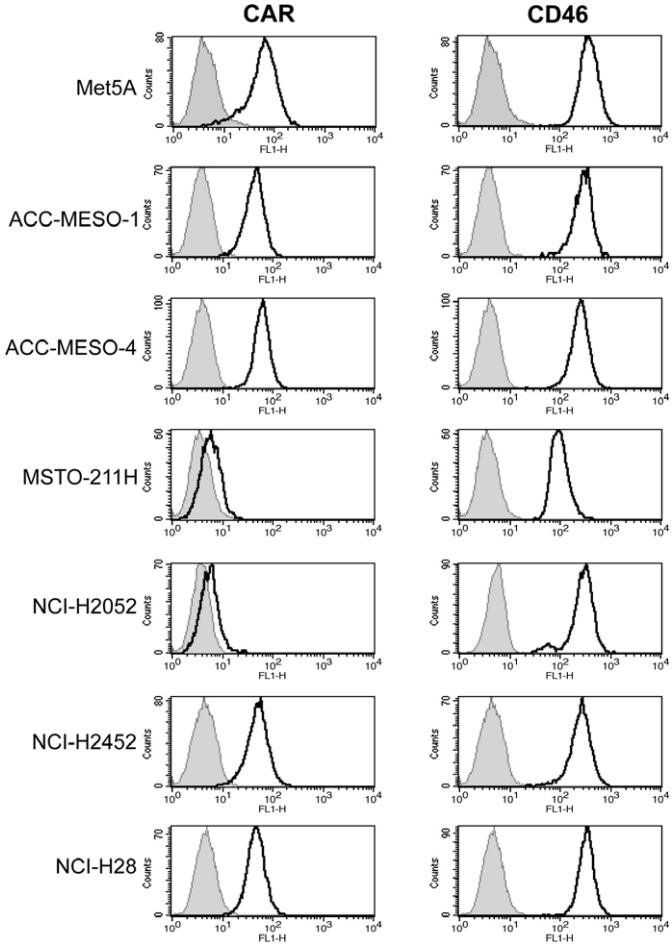

Fig. 1. Cell surface expression of coxsackie virus/adenovirus receptor (CAR) and CD46 in human mesothelioma cells. The expression of CAR (adenovirus serotype 5 [Ad5] receptor) and CD46 (Ad35 receptor) were evaluated in Met5A immortalized human mesothelial cells and six human mesothelioma cell lines (ACC-MESO-1, ACC-MESO-4, MSTO-211H, NCI-H2052, NCI-H2452, and NCI-H28) by flow cytometry. Open traces represent signals, and shaded traces represent negative controls.

CMV promoter-driven EGFP marker gene cassette in the adenoviral backbone to enable the monitoring of the presence and spread of the adenovirus through fluorescence expression.

Validation of fiber-modified adenoviruses. To investigate whether the fiber-modified adenoviruses achieve enhanced infectivity in mesothelioma cells, we first compared the infectivity of replication-defective adenoviruses with the wild-type Ad5 fiber (Ad843) or the Ad5/F35-modified fiber (Ad844). As shown in Figure 3(a), CAR-low MSTO-211H and NCI-H2052 cells were infected more efficiently with Ad844 (Ad5/F35 fiber) compared to Ad843 (Ad5 fiber), 6.53 *vs* 23.08-fold, respectively. This suggests that the Ad35 fiber modification confers enhanced cellular susceptibility to Ad5-based adenovirus in CAR-low mesothelioma cell lines. In addition, compared to NMC, the fiber-modified Ad844 infects mesothelioma cells more efficiently than Ad843, indicating Ad35 fiber modification confers tumor-selective enhancement of infectivity with adenoviruses.

We next examined the amount of viral progeny that was produced in mesothelioma cells after infection with the MDK-regulated oncolytic adenoviruses. When normalized to the baseline value in NMC, both oncolytic adenoviruses replicate selectively, and efficiently produce viral progeny in all mesothelioma cells (Fig. 3b). Among them, fiber-modified Ad889 showed much higher levels of virus production compared to Ad888 in low CAR-expressing MSTO-211H (3.7-fold) and NCI-H2052 (9.1-fold) cells. These findings indicate that enhanced infectivity by Ad35 fiber modification contributes largely to increase the virus progeny production of fiber-modified oncolytic adenoviruses especially in CAR-low cells. Furthermore, the fiber-modified Ad889 caused higher or comparable levels of virus progeny production even in CAR-high cells (ACC-MESO-1, ACC-MESO-4, NCI-H2452, and NCI-H28), which is consistent with the higher levels of infectivity of the fiber-modified viruses seen in these cell lines (Fig. 3a).

Oncolytic efficiency of MDK promoter-regulated oncolytic adenoviruses in normal and mesothelioma cell cultures. To investigate tumor-specific cytotoxicity, non-malignant cells (NMC, Met5A, and fibroblasts) and six malignant mesothelioma cell lines (ACC-MESO-1, ACC-MESO-4, MSTO-211H, NCI-H2052, NCI-H2452, and NCI-H28) were infected with adenoviruses at various MOIs. After 8 days, the oncolytic efficiency of the viruses was examined by staining adherent viable cells with crystal violet. As shown in Figure 4, both replication-defective adenoviruses, Ad843 and Ad844, showed no apparent cell death at an MOI of up to 1000 in all cell lines tested. In contrast, both oncolytic adenoviruses Ad888 and Ad889 caused dose-dependent cytolysis in all mesothelioma cell lines, although sensitivity varied between cell lines (Fig. 4). In mesothelioma cell lines, the fiber-modified Ad889 caused more extensive or comparable cell death compared to Ad888, which is consistent with the higher level of CD46 expression seen in most of these cell lines (Fig. 1). Of note, in CAR-low MSTO-211H and NCI-H2052 cells, Ad889 caused cell death at a 10-fold lower dose than Ad888 (MOI of 1 *vs* 10). In addition, in CAR-high ACC-MESO-4 cells, Ad889 caused cell death at a more than 10-fold lower dose than Ad888 (MOI of 0.1 *vs* 1). In contrast, Ad889 showed no apparent death in NMC, Met5A, or fibroblasts in which MDK promoter is not activated[21] even at a high MOI of 1000. This defines a therapeutic window, ranging between three and four orders of magnitude, for Ad889 cytotoxicity in mesothelioma cell lines compared to normal cells. These findings indicate that fiber modification with the CD46 binding domain confers enhanced tumor-specific cytotoxicity on MDK promoter-regulated oncolytic Ad888 in mesothelioma cell lines.

Viral dose- and time-dependency of oncolysis by MDK promoter-regulated oncolytic adenoviruses in normal and mesothelioma cell cultures. The cytotoxicity of the adenoviruses was assayed by infecting replicate cell cultures with Ad888 or Ad889 at various MOIs. At serial time points thereafter, the number of surviving cells was analyzed using a colorimetric method. As shown in Figure 5, neither Ad888 nor Ad889 caused any significant cytocidal effect in NMC and Met5A cells, but did cause varying degrees of viral dose- and time-dependent cytotoxicity in mesothelioma cell lines. In CAR-low MSTO-211H and NCI-H2052 cells, fiber-modified Ad889 caused significantly more extensive cell death than Ad888 with the wild-type Ad5 fiber, as expected. However, in these cells, Ad888 also caused extensive cell death at an MOI of 10 (Figs 4,5), suggesting that CAR deficiency is outweighed by other factors in these cell lines. Furthermore, in CAR-high cells, fiber-modified Ad889 caused comparable (ACC-MESO-1) or more extensive (ACC-MESO-4) cell death compared to Ad888, which is consistent with the levels of infectivity and virus progeny production of the fiber-modified viruses seen in these cell lines (Fig. 3). Taken together, Ad35 fiber modification enhanced the overall transduction efficiency and

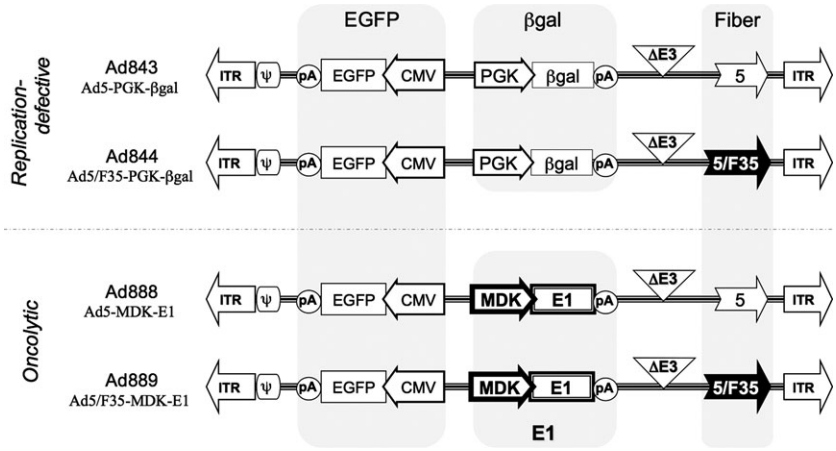

Fig. 2. Schematic representation of recombinant adenovirus vectors. The top two replication-defective adenoviruses contain a phosphoglycerate kinase-1 (PGK) promoter-driven βgal gene with adenovirus serotype 5 (Ad5) fiber (Ad843) or Ad5/F35-modified (Ad844) fiber. The bottom two oncolytic adenoviruses contain a midkine (MDK) promoter-regulated E1 gene expression cassette with Ad5 fiber (Ad888) or Ad5/F35-modified fiber (Ad889). All vectors have an independent CMV promoter-driven enhanced green fluorescent protein (EGFP) gene cassette in the adenoviral backbone. ΔE3, adenoviral E3 gene-deleted; ITR, adenovirus inverted terminal repeat sequence; pA, polyadenylation signal; ψ, packaging signal.

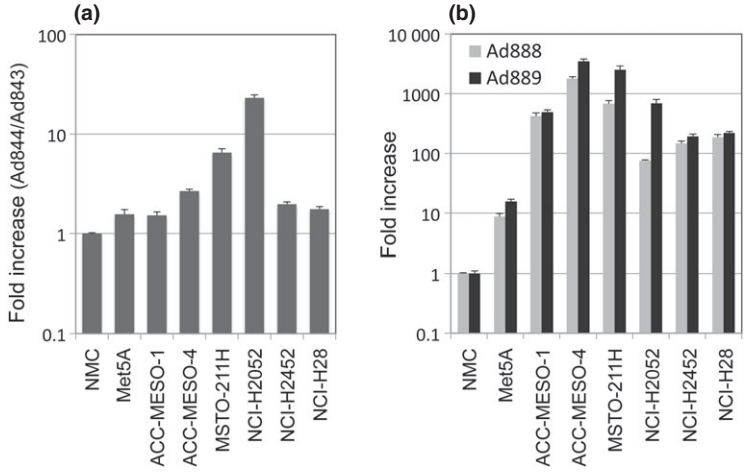

Fig. 3. Validation of fiber-modified adenoviruses. (a) Infectivity of adenoviruses with adenovirus serotype 5 (Ad5) fiber or Ad5/F35-modified fiber. Normal mesothelial cells (NMC), Met5A, and six mesothelioma cell lines (ACC-MESO-1, ACC-MESO-4, MSTO-211H, NCI-H2052, NCI-H2452, and NCI-H28) were infected with serially diluted replication-defective adenoviruses, Ad843 (Ad5 fiber) or Ad844 (Ad5/F35 fiber). Twenty-four hours later, enhanced green fluorescent protein titers were determined by flow cytometry, compared between Ad843 and Ad844 (Ad844/Ad843). Data were normalized to the baseline value in NMC, and shown as the mean ± SD calculated from triplicates. (b) Virus progeny production of oncolytic adenoviruses with Ad5 fiber or modified fiber. Cells were infected with midkine promoter-regulated oncolytic adenoviruses Ad888 (Ad5 fiber) or Ad889 (Ad5/F35 fiber) at an MOI of 100. Forty-eight hours after infection, cells and media were harvested to determine the viral titer on HEK293 cells. Virus production levels were normalized to the baseline value in NMC, and shown as the mean ± SD calculated from triplicates.

thus increased the selective cytotoxicity in most of these mesothelioma cells.

Antitumor efficacy of adenoviruses in a malignant mesothelioma xenograft model. We examined *in vivo* antitumor efficacy in an s.c. MSTO-211H xenograft model pre-established in athymic nude mice, which received two intratumoral injections of PBS, Ad844 (replication-defective adenovirus with fiber modification), Ad888 (oncolytic with Ad5 fiber), or the fiber-modified oncolytic Ad889. As shown in Figure 6, treatment with Ad888 resulted in a 40.8–43.9% reduction in tumor volume (1159.2 ± 321.7 mm^3) compared to PBS treatment (1958.5 ± 437.5 mm^3) or Ad844 (2066.3 ± 475.6 mm^3) by day 49 post-virus injection. The fiber-modified Ad889 showed a further, significant reduction in tumor volume (466.3 ± 175.6 mm^3) by day 49 and a 59.8% reduction in tumor volume compared to Ad888 ($P < 0.05$). These data indicate that the Ad35 fiber modification of MDK-targeted adenoviruses enhances the antitumor efficiency of MDK-targeted oncolytic virotherapy in experimental cancer models *in vivo*.

Discussion

Tumor specificity and viral infectivity are critical to the success of oncolytic virotherapy. Even when replicating viruses are used, viral infectivity of target cells is a critical determinant of therapeutic efficacy. We recently showed[21] that the MDK promoter confers tumor-selective transcriptional targeting on Ad5-based oncolytic adenoviruses, which proved highly effective in malignant mesothelioma models. In this previous study, we showed that MDK-regulated oncolytic adenoviruses exert a robust tumor-specific cytotoxicity in ACC-MESO-4 mesothelioma cells, and complete regression of s.c. tumor xenografts in athymic mice. This striking result may reflect the high permissivity of ACC-MESO-4 cells for MDK-regulated adenovirus infection, supported by the fact that ACC-MESO-4 cells showed the highest observed levels of MDK and CAR expression among a panel of six mesothelioma cell lines tested in the current study. However, in MSTO-211H and NCI-H2052 cells, which show high MDK but very low CAR expression, oncolytic effects of MDK-regulated oncolytic

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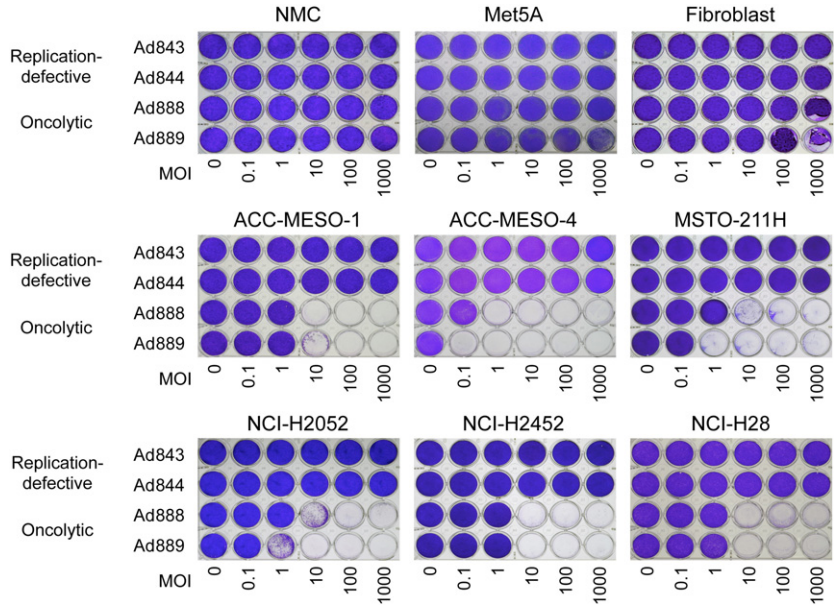

Fig. 4. Oncolytic efficiency of midkine promoter-regulated oncolytic adenoviruses in normal and mesothelioma cell cultures. Non-malignant human cells (normal mesothelial cells [NMC], Met5A, and fibroblasts), and six human mesothelioma cell lines (ACC-MESO-1, ACC-MESO-4, MSTO-211H, NCI-H2052, NCI-H2452, and NCI-H28) were infected with adenoviruses at an MOI ranging from 0.1 to 1000. Crystal violet staining of viable cells was used to evaluate oncolytic activity at 8 days postinfection.

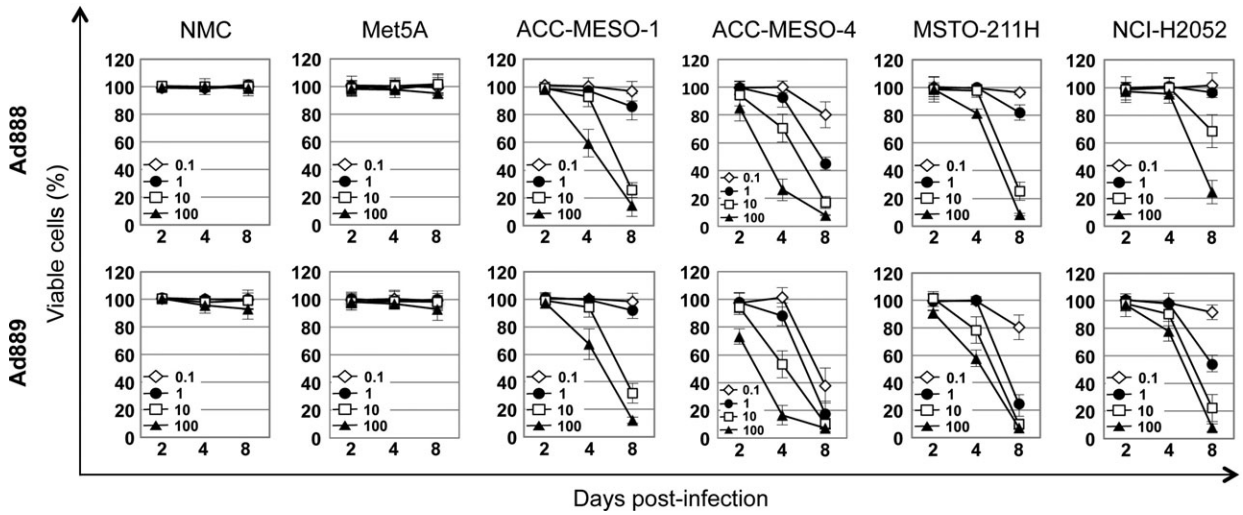

Fig. 5. Viral dose and time dependency of oncolysis by midkine promoter-regulated oncolytic adenoviruses in normal and mesothelioma cell cultures. Cells were cultured in multiple replicate 96-well plates and infected with Ad888 or fiber-modified Ad889, at MOIs from 0.1 to 100. On day 2, day 4, and day 8, the number of surviving cells was analyzed by a colorimetric method. Data shown are the mean ± SD calculated from triplicates.

adenovirus with the wild-type Ad5 fiber were subtherapeutic relative to ACC-MESO-4 cells.[21]

In the present study, we found that CAR expression levels varied significantly in six human mesothelioma cell lines in which the MDK promoter is highly activated. In contrast, CD46 was strongly expressed in all of these cell lines (Fig. 1). Accordingly, we investigated the use of the Ad35 fiber for modification of physical binding tropism (transductional targeting) combined with MDK promoter regulation (transcriptional targeting) of oncolytic Ad5 vectors. This dual-targeted adenovirus (Ad889) showed tumor cell-specific replication and cytotoxicity, but spared normal cells (Figs 4,5). The dual-targeted virus also showed enhanced antitumor efficacy against CAR-low mesothelioma cells both *in vitro* and *in vivo*.

Unexpectedly, Ad35 fiber modification of the adenoviruses conferred more or comparable levels of infectivity, virus progeny production, and cytotoxicity in all mesothelioma cells (Figs 3–5). This may be caused by the higher level of CD46 expression in these cell lines, compared to CAR. Consequently, Ad35 fiber-modified, transcriptionally targeted oncolytic adenoviruses would be an ideal platform of virotherapy for malignant mesothelioma. In clinical situations, however, it will be feasible to evaluate CAR/CD46 and MDK expression in tumor specimens for selecting suitable oncolytic adenoviruses. Our MDK promoter-regulated oncolytic adenoviruses with wild-type Ad5 fiber and Ad5/F35 fiber may achieve tailor-made virotherapy for various but specific types of cancers with upregulated MDK expression.

Only a few studies have reported oncolytic adenoviruses with Ad35 fiber modifications. Sova *et al.*[35] and Ni *et al.*[38] constructed a capsid-modified Ad5-based oncolytic adenovirus containing short-shafted entire fibers derived from Ad35

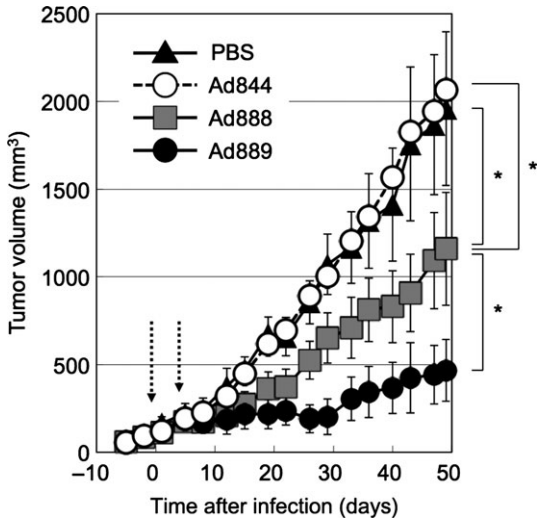

Fig. 6. *In vivo* antitumor effect of oncolytic adenoviruses in a malignant mesothelioma xenograft model. MSTO-211H tumors were grown s.c. in nude mice to approximately 5–6 mm in diameter and injected with 5×10^7 transducing units of replication-defective Ad844, oncolytic Ad888, fiber-modified oncolytic Ad889, or PBS as a control ($n = 11$/group), on days 0 and 4. Tumor volumes were measured twice a week, and data shown are the mean ± SD. *$P < 0.05$.

and showed efficient infection of lung, liver, colon, prostate, ovarian, and cervical cancer cells. Similarly, Hoffmann *et al.* constructed an Ad5-based oncolytic adenovirus with the fiber shaft and knob domain of Ad35 and showed that the chimeric oncolytic vector has a significantly higher transduction efficiency of glioblastoma,[39] sarcoma,[40] and melanoma[41] cells than the Ad5 vector. Furthermore, Yu *et al.*[32,34] constructed a chimeric oncolytic adenovirus in which the Ad5 fiber knob was replaced with the Ad35 fiber knob, and showed enhanced transduction compared to that observed with the Ad5 fiber knob in esophageal, pancreatic, and breast cancer cells. Our study is the first to demonstrate an enhanced therapeutic effect of an Ad35 fiber-modified, transductionally targeted oncolytic adenovirus in malignant mesothelioma models.

To evaluate the *in vivo* therapeutic efficacy of fiber-modified Ad889 in CAR-low mesothelioma cells, it was necessary to use MSTO-211H cells, as NCI-H2052 cells are not tumorigenic in nude mice. However, in MSTO-211H cells, Ad888 with the wild-type Ad5 fiber also caused extensive cell death at an MOI of 10 (Figs 4,5). Our previous work[21] showed that high-dose MDK-regulated oncolytic adenoviruses with the wild-type Ad5 fiber did achieve significant tumor reduction in MSTO-211H xenograft mouse models. Taken together, the results from both studies suggest that CAR deficiency can be outweighed by other factors, such as coreceptors (e.g., integrins), the strength of the MDK-promoter activity, apoptotic death mechanisms, or innate immune responses, in MSTO-211H cells. Further enhancement of therapeutic efficacy may also be possible by incorporating suicide gene therapy, as we previously demonstrated[21] that an MDK promoter-regulated oncolytic adenovirus armed with an HSV-TK suicide gene showed increased *in vivo* antitumor activity.

In conclusion, in the present study we have shown that Ad35 fiber modification of MDK-targeted oncolytic adenovirus vectors can increase viral infectivity and confer enhanced transduction efficiency and antitumor efficacy in malignant mesothelioma cells especially in which CAR is downregulated. As MDK and CD46 are both highly upregulated in a variety of cancers, including malignant mesothelioma, our dual-targeted oncolytic adenovirus may represent a highly promising general strategy for combined transcriptional and transductional targeting of oncolytic virotherapy.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

Ad5	adenovirus serotype 5
Ad35	adenovirus serotype 35
Ad843	Ad5-PGK-βgal adenoviral vector
Ad844	Ad5/F35-PGK-βgal adenoviral vector
Ad888	Ad5-MDK-E1 adenoviral vector
Ad889	Ad5/F35-MDK-E1 adenoviral vector
CAR	coxsackie virus/adenovirus receptor
EGFP	enhanced green fluorescent protein
MDK	midkine
MOI	multiplicity of infection
NMC	normal mesothelial cells
PGK	phosphoglycerate kinase-1
TU	transducing unit

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