Correlation between Adenovirus-neutralizing Antibody Titer and Adenovirus Vector-mediated Transduction Efficiency Following Intratumoral Injection

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Abstract. *Pre-existing anti-adenovirus neutralizing antibodies (AdNAbs) are a major barrier in clinical gene therapy using adenovirus vectors; however, the transduction profile of adenovirus vectors in the presence of AdNAbs following intratumoral injection has not been fully examined, although such vectors are often intratumorally injected in clinical studies. In this study, we evaluated the correlation between the titer of AdNAbs in the serum and the transduction profiles in the tumor and the liver following intratumoral administration into mice possessing various titers of AdNAbs. Adenovirus vector-mediated transduction in the tumor was inhibited by AdNAbs; however, when the titer of AdNAbs was less than 200, the levels of inhibition in the transduction efficiencies within the tumor ranged from approximately 2- to 100-fold. A more than 2500-fold reduction of adenovirus vector-mediated transduction was found in most of the mice when the titers of AdNAbs were >200. On the other hand, the transduction efficiencies in the liver were largely reduced almost to the levels of the mock-transduced mice even at the low titers of AdNAbs. These results provide crucial information for the clinical use of adenovirus vectors.*

Replication-incompetent adenovirus vectors are widely used in not only gene therapy studies, but also in basic research due to their numerous advantages as a gene delivery vehicle. For example, adenovirus vectors efficiently transduce both dividing and non-dividing cells *in vitro* and *in vivo*. A foreign gene of relatively large size can be included in the adenovirus vector genome. Higher titers of adenovirus vectors can be obtained compared with other viral vectors (1, 2). Furthermore, various types of recombinant oncolytic adenoviruses, which replicate specifically in tumor cells, leading to efficient tumor cell killing, have been developed and used in clinical trials.

However, a major limitation of gene therapy using adenovirus vectors, including virus therapy using oncolytic adenoviruses, is that adenovirus vector-mediated transduction is largely disturbed by pre-existing anti-adenovirus neutralizing antibodies (AdNAbs) (3-6). Fifty-seven adenovirus serotypes have now been identified and classified into six species (7-9). Among these 57 serotypes, adenovirus serotype 5 (Ad5) is the the one most commonly used to construct vectors. More than 80% of adults have been naturally infected with and are sero-positive for Ad5 (10-13). Ad5-neutralizing antibodies mainly recognize hexon and/or fiber protein, leading to a significant reduction in the transduction efficiencies, especially when adenovirus vectors are intravascularly administered (3-5).

In clinical trials, adenovirus vectors are often directly injected into the tumors (14-16). The degree to which pre-existing AdNAbs inhibit adenovirus vector-mediated transduction in the tumor following intratumoral administration is controversial (17-21). Several studies have demonstrated that transduction with an adenovirus vector in the tumor was not significantly inhibited following intratumoral administration in mice pre-immunized with adenoviruses (17-20). On the other hand, Vlachaki *et al.* demonstrated a reduction in the transduction efficiencies of an adenovirus vector following intratumoral administration in pre-immunized mice (21). In addition, previous studies have exhibited only a difference in the averages of the transduction efficiencies of an adenovirus vector between a

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group of naïve and a group of pre-immunized mice (17-22). The correlation between titers of AdNAbs and transduction efficiencies of adenovirus vector in individual mice has not been evaluated, although titers of AdNAbs in human serum vary widely among individuals (10, 12, 13).

In this study, in order to examine the correlation between the titer of AdNAbs in serum and transduction profiles in the tumor and liver following intratumoral administration, mice possessing various titers of AdNAbs were prepared by intravenous administration of various doses of an adenovirus vector. A luciferase-expressing adenovirus vector was intratumorally injected into tumor-bearing mice with various titers of AdNAbs. Luciferase expression and adenovirus vector genome copy number in the tumor and liver were evaluated.

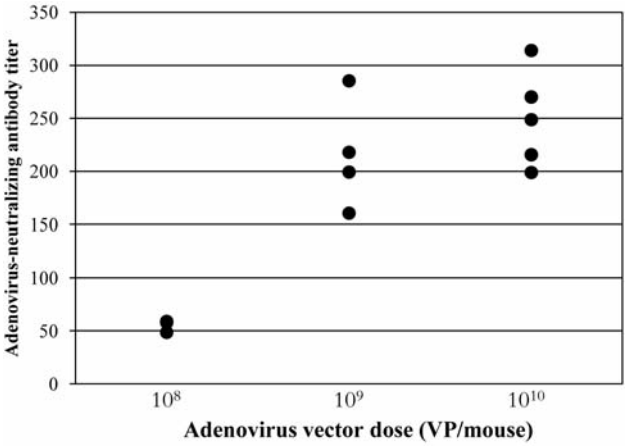

Figure 1. *Adenovirus neutralizing antibody (AdNAb) titer in the serum of pre-immunized mice. Immunocompetent mice were immunized via intravenous injection of the indicated doses of null vector (n=3-5). On day 19 post-immunization, just before administration of AdRGD-L2, which is a luciferase-expressing fiber-mutant adenovirus vector containing an RGD peptide in the HI loop of fiber knob, the serum samples were collected and subjected to the analysis of AdNAb titer by neutralizing assay. Each plot in the graph indicates an individual mouse.*

Materials and Methods

Cells. A549 cells (a human lung adenocarcinoma epithelial cell line, obtained from American Type Culture Collection (ATCC), Manassas, VA, USA; CCL-185) were cultured with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and antibiotics. B16 cells (B16BL6, mouse melanoma, kindly provided by Dr S. Nakagawa (Osaka University, Japan)) were cultured with minimum essential medium containing 10% FBS and antibiotics.

Adenovirus vectors. An adenovirus vector containing no transgene expression cassette (Ad-null) was previously constructed (23). A luciferase-expressing conventional adenovirus vector, Ad-L2, and a fiber-mutant adenovirus vector containing an RGD (Arg-Gly-Asp) peptide in the HI loop of fiber knob, AdRGD-L2, were previously constructed (24). Determination of the virus particle titers was accomplished spectrophotometrically by the method of Maizel *et al*. (25)

Pre-immunization with an adenovirus vector. For pre-immunization of mice with adenovirus, 7-week-old female C57BL/6 mice obtained from Nippon SLC (Hamamatsu, Japan) were intravascularly injected with Ad-null at various doses ranging from 5×10^7 to 1×10^{10} vector particles (VP)/mouse. Determination of AdNAb titers in serum was performed 19 days after immunization (see below). The protocol of the experimental procedures was approved by the Animal Welfare and Animal Care Committee of Osaka University and National Institute of Biomedical Innovation (Osaka, Japan).

Luciferase assay in vivo. B16 cells (5×10^5 cells) were intradermally inoculated into the mouse abdomen 9 days after pre-immunization with Ad-null (on day 9) as described above. On day 19, when the tumor diameter exceeded approximately 5 mm, 50 μl of AdRGD-L2 (1×10^9 VP) was intratumorally injected. Two days following injection with AdRGD-L2, the tumors and livers were recovered and homogenated as previously described (26). Luciferase productions were determined using a luciferase assay system (PicaGene 5500; Toyo Inki Co., Tokyo, Japan).

Determination of AdNAb titers in serum. The titers of AdNAbs in serum isolated from pre-immunized mice were determined by analyzing the ability of AdNAbs to inhibit transduction with Ad-L2 in A549 cells, as previously described (27). Briefly, serum samples were collected by retro-orbital bleeding on day 19. A549 cells were seeded on a 96-well plate at 1×10^4 cells/well, the serum was subjected to a serial doubling dilution and then added to each well. As a control, serum collected from naïve mice was used. The cells were then transduced with Ad-L2 at 500 VP/cell for 1.5 h. The final dilution factors of mouse serum were from 1/20 to 1/3200. After 48-h incubation, luciferase production was determined using a luciferase assay system (PicaGene LT2.0, Toyo Inki Co.), as described above. AdNAb titer was determined by the dilution of serum that resulted in a 50% inhibition of luciferase production compared with the control serum.

Real-time PCR analysis of adenovirus vector genome copy numbers. Total DNA, including the adenovirus vector genome, was isolated from the tumor and liver by an automatic nucleic acid isolation system (NA-2000; Kurabo Industries, Osaka, Japan) two days after administration of AdRGD-L2. The copy numbers of adenovirus genomic DNA in the tumor and the liver were quantified with the TaqMan fluorogenic detection system (StepOnePlus™ Real Time PCR System; Applied Biosystems, Foster City, CA, USA) as previously described (28).

In vivo imaging of adenovirus vector-mediated luciferase expression. Mice were pre-immunized with 5×10^7 VP of Ad-null. B16 cells were intradermally inoculated into the non-immunized and pre-immunized mice 9 days after immunization as described above. AdRGD-L2 was intratumorally injected on day 19 after inoculation. Before imaging, luciferin (Promega, Madison, WI, USA) suspended in sterile phosphate-buffered saline (PBS) was intraperitoneally injected (2 mg/mouse/200 μl). Several minutes later, luminescence was detected using NightOWL LB983 (Belthold, Bad Wildbad, Germany). Luciferase expression was measured on 2, 4, and 7 days following intratumoral administration.

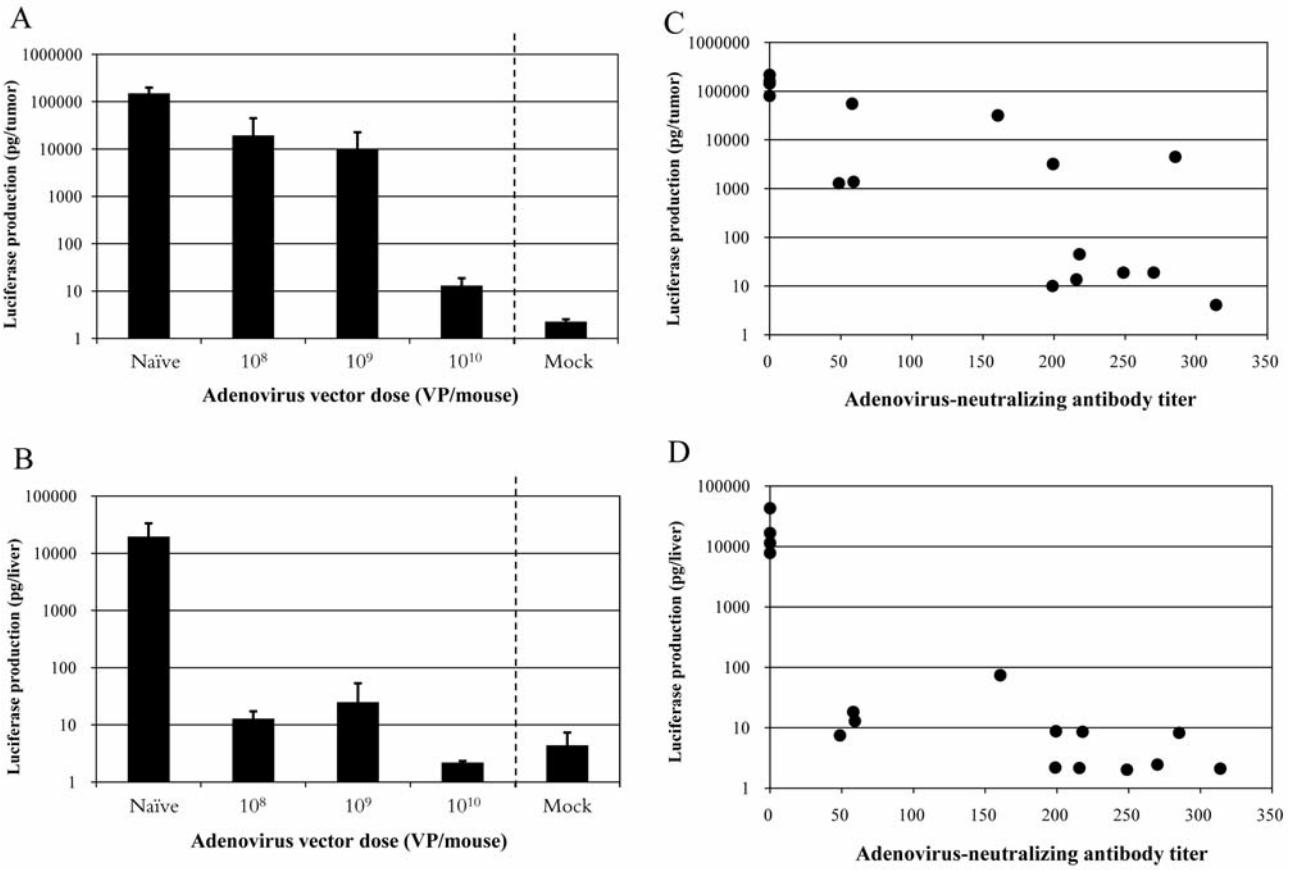

Figure 2. *Luciferase expression in the tumor after intratumoral administration of AdRGD-L2, which is a luciferase-expressing fiber-mutant adenovirus vector containing an RGD peptide in the HI loop of fiber knob, into the non-immunized and pre-immunized mice. AdRGD-L2 (1×10^9 vector particles) was intratumorally administered on day 19 after pre-immunization. Two days later, luciferase expression in the tumor (A) and liver (B) was measured by luciferase assay (n=3-5). Correlation between the AdNAb titer in serum and luciferase production in the tumor (C) and liver (D) of an individual mouse. Each plot indicates data from an individual mouse.*

Results

Titers of AdNAbs in serum following intravenous administration of various doses of an adenovirus vector. Mice possessing various titers of AdNAbs in serum were prepared by administering different doses of Ad-null. The serum of naïve mice did not affect the transduction efficiencies of the adenovirus vector in A549 cells (data not shown). All serum samples from the pre-immunized mice exhibited the neutralizing activity of the adenovirus vector (Figure 1). The titers of AdNAbs appeared to depend on the injected doses of Ad-null, although the titers of AdNAbs were different between the individual mice. The titers of AdNAbs in the serum of pre-immunized mice ranged from 49 to 314.

Transduction efficiencies of an adenovirus vector in the tumors of pre-immunized mice following intratumoral administration. In order to examine the transduction efficiencies of an adenovirus vector following intratumoral administration into pre-immunized mice, a luciferase-expressing adenovirus vector, AdRGD-L2, which efficiently transduces coxsackievirus-adenovirus receptor (CAR)-negative cells *via* interaction between an RGD peptide in the fiber knob and αv-integrins on the cell surface, was directly injected into the subcutaneous tumors (B16 melanoma) of mice pre-immunized with Ad-null. In the tumors of mice pre-immunized with 1×10^8 VP and 1×10^9 VP of Ad-null, the transduction efficiencies were reduced by 8-fold and 15-fold, compared with those in the tumors of non-immunized mice, respectively (Figure 2A). However, an approximately 11600-fold reduction in the transduction efficiencies was found in the tumors of mice pre-immunized with 1×10^{10} VP of Ad-null. The titers of AdNAbs in the serum and the transduction efficiencies of AdRGD-L2 in the tumors of individual mice are plotted in Figure 2C. The data in Figure 2C clearly show that in all mice in which the titer of AdNAbs was lower than 200 (4 out of 4 mice), the reductions in transduction efficiencies in the tumor were

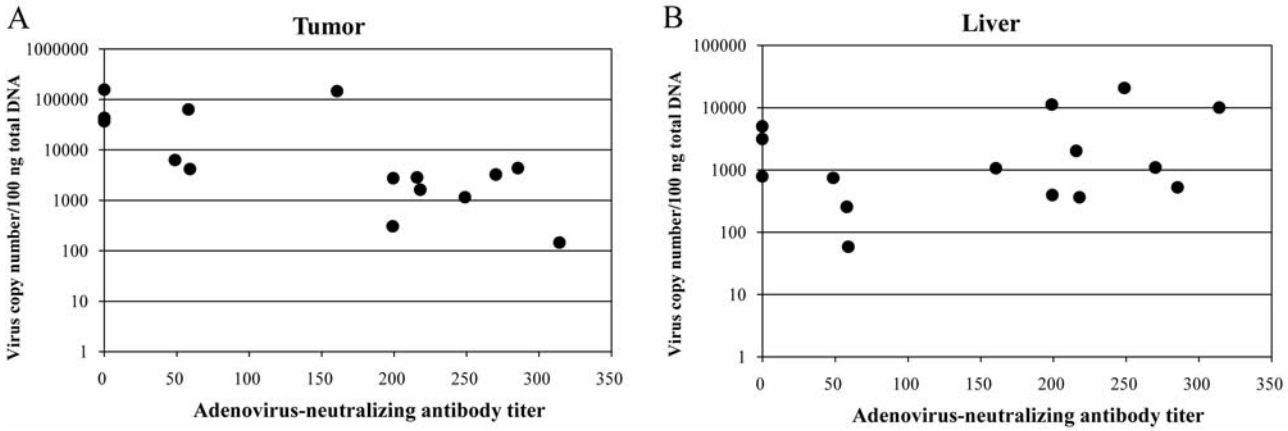

Figure 3. *Correlation between the adenovirus neutralizing antibodies (AdNAb) titer in serum and the copy numbers of adenovirus vector genome in the tumor (A) and liver (B) after intratumoral administration of AdRGD-L2, which is a luciferase-expressing fiber-mutant adenovirus vector containing an RGD peptide in the HI loop of fiber knob. Two days after intratumoral administration, the tumor and liver were harvested, and the copy numbers of the adenovirus vector genome were measured by quantitative TaqMan PCR assay. Each plot indicates data from an individual mouse.*

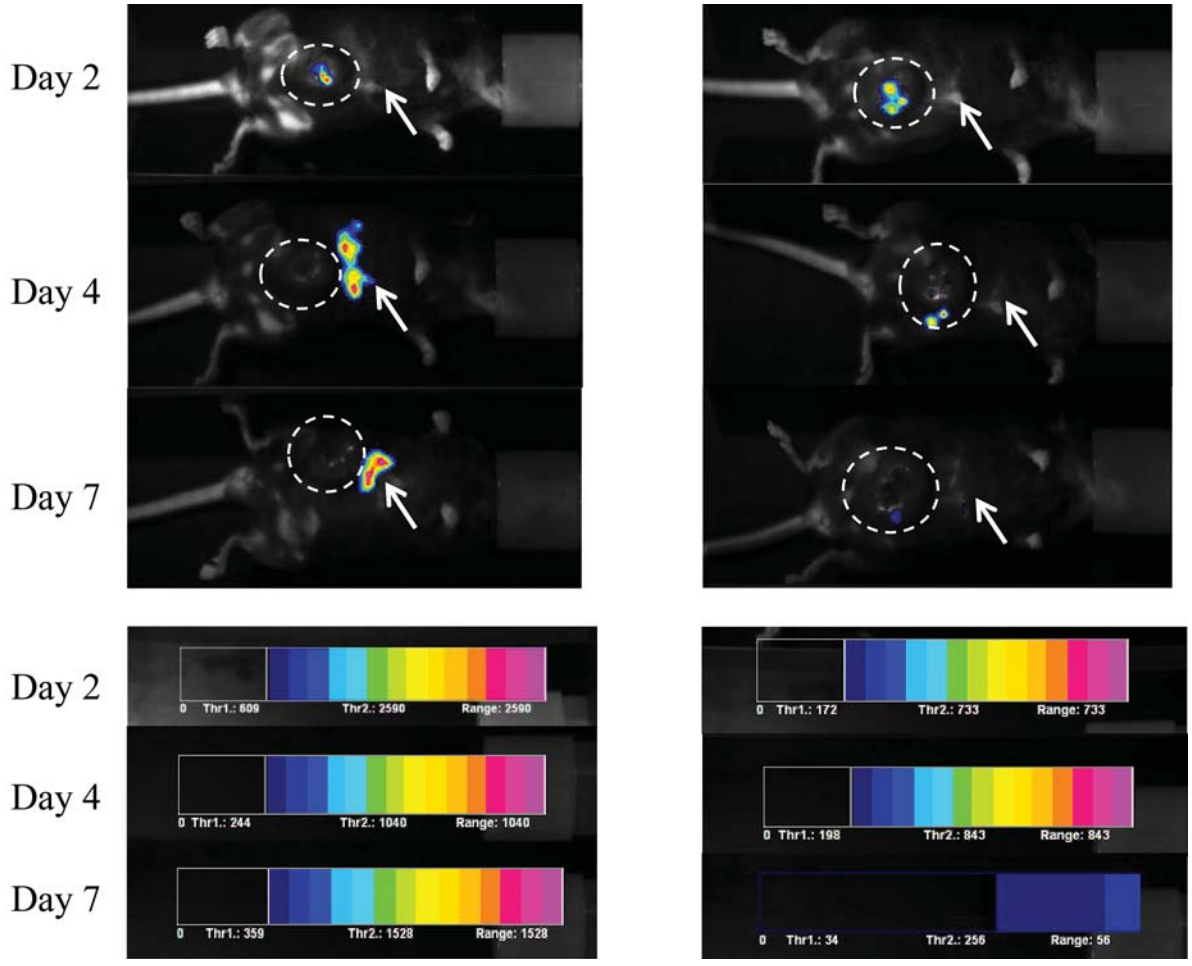

Figure 4. *Time-course analysis of luciferase expression by in vivo imaging. For pre-immunization, mice were intravenously injected with null vector (5×10^7 vector particles (VP)/mouse) (day 0). On day 19, AdRGD-L2 (1×10^9 VP/mouse), which is a luciferase-expressing fiber-mutant adenovirus vector containing an RGD peptide in the HI loop of fiber knob, was intratumorally injected. Luciferase expressions in a non-immunized mouse (A) and a pre-immunized mouse (B) were measured at 2, 4, and 7 days after intratumoral injection. The tumors of the mice are indicated by the area surrounded by a dotted line, and the liver is indicated by a white arrow. Color scale rulers for each examination are shown below the images.*

within the range of approximately 2- to 100-fold compared with the average transduction efficiencies in the tumors of non-immunized mice. However, a much larger reduction in the transduction efficiencies was found in mice with a titer of AdNAbs >200 than in mice with a titer of AdNAbs <200. Six out of eight mice with titers >200 exhibited a more than 2500-fold reduction in the transduction efficiencies in the tumor compared with the non-immunized mice. These results indicate that the adenovirus vector still possessed transduction activity in the tumor following intratumoral injection of the low titer of AdNAbs, but the transduction efficiencies in the tumor significantly reduced as the titer of AdNAbs increased, and especially when the titers were above 200.

Transduction efficiencies of adenovirus vector in the livers of pre-immunized mice following intratumoral administration. In order to evaluate the transduction efficiencies in the liver of pre-immunized mice following intratumoral administration of AdRGD-L2, the liver was harvested and its luciferase activity was measured. Several groups, including our own, demonstrated that adenovirus vector which was intratumorally injected leaked from the injected site and drained into the systemic circulation, resulting in efficient accumulation and transduction in the liver (21, 26, 29). As previously reported, a high level of luciferase expression was found in the livers of non-immunized mice (Figure 2B). In contrast, in all pre-immunized mice, the transduction efficiencies in the liver were dramatically reduced almost to those in the mock-transduced mice, even when pre-immunized with $1x10^8$ VP of Ad-null. Figure 2D shows the correlation plots between the titers of AdNAbs in the serum and the transduction efficiencies in the liver. The correlation pattern of the liver was largely different from the one observed on the tumor (Figure 2C). The transduction efficiencies in the liver were almost at background levels even in the animals with an AdNAb titer <200. These results indicated that adenovirus vector-mediated transduction in the liver was inhibited by AdNAbs to a greater degree than that in the tumor.

Tissue accumulation of adenovirus vector in the pre-immunized mice following intratumoral administration. In order to examine whether AdNAb titers in serum affect the tissue distribution of adenovirus vector following intratumoral administration, the copy numbers of adenovirus vector genome in the tumor and un the liver were evaluated by real-time PCR analysis two days after intratumoral administration of AdRGD-L2. In the tumor, similar to the correlation profile between the transduction efficiencies and the AdNAb titers, the copy numbers of adenovirus vector genome were reduced as the titer of AdNAbs increased (Figure 3A). However, the inhibitory effects of the AdNAbs on the adenovirus vector genome levels in the tumor were smaller than those on the transduction efficiencies. For example, when the titer of AdNAbs was 218, a 48-fold reduction in the copy number of the adenovirus vector genome in the tumor was found, in spite of the 2857-fold reduction in the transduction efficiency in the tumor, compared with the average of the adenovirus genome copy number in the non-immunized mice. Liver accumulation of the adenovirus vector genome did not appear to be inhibited by the AdNAbs (Figure 3B). These results indicated that AdNAbs inhibited the tissue accumulation of the adenovirus vector in the organs less effectively than they did the transduction efficiencies.

Effect of pre-immunization on the time-course of adenovirus vector-mediated transgene expression. In order to examine whether pre-immunization with Ad-null affects the time-course of adenovirus vector-mediated transgene expression, we monitored the luciferase expression in the tumor and in the liver after intratumoral administration in non-immunized and pre-immunized mice by *in vivo* imaging analysis. In the non-immunized mice, luciferase expression in the tumor was detected on day 2, but had diminished by day 4. On the other hand, luciferase expression in the liver of non-immunized mice was not clearly detected on day 2, however, luciferase expression was apparent on days 4 and 7 (Figure 4A). In the pre-immunized mice, luciferase expression was also detected on day 2 (Figure 4B); however, the intensity of luciferase expression was weaker than that in the non-immunized mice. On days 4 and 7, there was no detectable level of luciferase expression in the tumors of pre-immunized mice. The livers of the pre-immunized mice also did not exhibit a detectable level of luciferase expression at these time points. In another pre-immunized mouse, luciferase expression in both the tumor and liver was below the detectable level throughout the period examined (data not shown).

Discussion

Ad5 is one of the most common pathogens leading to respiratory diseases, and the ubiquity of this pathogen has led to a high seroprevalence of Ad5 in adults in the general population. High titers of AdNAbs significantly inhibit adenovirus vector-mediated *in vivo* transduction, which is the most crucial hurdle to overcome in the clinical use of adenovirus vectors. However, the transduction profile of adenovirus vectors in pre-immunized mice, and especially the one following intratumoral injection of adenovirus vectors, has not been fully examined, despite the fact that such vectors are often directly administered to the tumor in clinical settings (14-16). The aim of this study was to examine the correlation between the titers of AdNAbs and the transduction profiles of an adenovirus vector following intratumoral administration.

Although high seroprevalence to Ad5 in adults in the general population has previously been reported (10-13), the titers of AdNAbs in human serum showed an extremely wide variation among different age groups and geographic regions, and the AdNAb titers in the seropositive human population ranged from 18 to 1000 or more (10, 12, 13). In this study, mice possessing various titers of AdNAbs with a range from 49 to 314 were produced by systemic administration of various doses of the adenovirus vector to mimic the variety of the AdNAb titers in humans. These titers of AdNAbs in the pre-immunized mice were considered to correspond to human titers within the normal range, although the method used here to determine the AdNAb titers has differed somewhat from previous studies.

The data of Figure 2 demonstrated that the transduction efficiencies of the adenovirus vector appeared to be inversely correlated with the titer of AdNAbs; however, when the titer was less than 200, the transduction efficiencies in the tumor were reduced approximately 2- to 100-fold. On the other hand, six out of eight mice in which the titer was more than 200 showed a more than 2500-fold reduction in the transduction efficiencies in the tumor. An AdNAb titer of 200, which would be in the normal range for general human populations, might be a threshold of efficient transduction following intratumoral administration of an adenovirus vector. It could be that when the AdNAb titer in serum is below a certain threshold, relatively efficient transduction in the tumor could be obtained following intratumoral administration. The AdNAb titer in the serum would be an important predictive factor for effective cancer gene therapy *via* intratumoral administration of an adenovirus vector, although further analysis of the correlation between the AdNAb titer and the transduction efficiency in the tumor is indeed necessary.

A reduced, but still relatively efficient transgene expression was found in the tumor when the titer of AdNAbs was less than 200. On the other hand, transduction with the adenovirus vector in the liver was completely blocked regardless of the titer of AdNAbs in the pre-immunized mice. These results indicated that an adenovirus vector in the bloodstream is susceptible to inhibition by AdNAbs. The efficient inhibition of adenovirus vector-mediated transduction by AdNAbs in the bloodstream would be preferable to enhance the safety of cancer gene therapy following intratumoral administration of an adenovirus vector, because unfavorable transgene expression in the liver would be avoided by AdNAbs without extensively disturbing transduction in the tumor.

To effectively overcome the inhibition by AdNAbs, several approaches, including covalent conjugation of polyethyleneglycol (PEG) (PEGylation) of adenovirus capsid proteins, adenovirus vector containing modified hexons, adenovirus vector composed of another serotype, and oncolytic adenovirus-loaded carrier cells, have been developed (4, 30-34). When the titer of AdNAbs is high enough to extensively inhibit the transduction, the approaches described above should be used to improve transduction efficiency.

In this study, the adenovirus vector genome copy numbers in the liver were not significantly reduced in the pre-immunized mice, in contrast to the significant reduction in transduction efficiency in the liver as described above. In addition, the reduction in the adenovirus vector copy number in the tumor was also of a smaller scale than that for the transduction efficiencies. At the present time, it is not clear why the adenovirus vector genome copy number was less affected by the AdNAbs in serum; however, the adenovirus vector associated with the AdNAbs might have been accumulated in the organs, but might not have been efficiently internalized; or intracellular trafficking of the adenovirus vector associated with the AdNAbs might have been inhibited by the AdNAbs. It is also possible that the adenovirus vector associated with the AdNAbs was taken-up by Kupffer cells in the liver, resulting in inefficient transduction. Kupffer cells are not readily susceptible to adenovirus vector-mediated transduction (35).

In conclusion, this study demonstrated that the transduction efficiencies of an adenovirus vector in the tumor largely depended on the titer of AdNAbs following intratumoral administration. When the titers of AdNAbs were below 200, relatively efficient transduction was still achieved although transgene expression was reduced by approximately 2- to 100-fold. Transduction in the tumor was largely blocked at AdNAb titers >200. These results should provide valuable information for the clinical use of adenovirus vectors.

Acknowledgements

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