

ORIGINAL ARTICLE

Changing the adenovirus fiber for retaining gene delivery efficacy in the presence of neutralizing antibodies

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Prior infection has primed most adult humans for a rapid neutralizing antibody (NAb) response when re-exposed to adenovirus. NAb induction can severely limit the efficacy of systemic re-administration of adenoviral gene therapy. We hypothesized that changing the fiber knob could overcome NAb. Immune-competent mice were exposed to serotype 5 adenovirus (Ad5)(GL), Ad5/3luc1, Ad5lucRGD or Ad5pK7(GL). Mice immunized with Ad5(GL) featured reduced intravenous Ad5(GL) gene transfer to most organs, including the liver, lung and spleen. Ad5(GL) gene transfer was affected much less by exposure to capsid-modified viruses. Anti-Ad5(GL) NAb blocked intravenous Ad5(GL) gene transfer to orthotopic lung cancer xenografts, whereas capsid-modified viruses were not affected. When gene

transfer to fresh cancer and normal lung explants was analyzed, we found that capsid-modified viruses allowed effective gene delivery to tumors in the presence of anti-Ad5(GL) NAb, whereas Ad5(GL) was blocked. In contrast, crossblocking by NABs induced by different viruses affected gene delivery to normal human lung explants, suggesting the importance of non-fiber-knob-mediated infection mechanisms. We conclude that changing the adenovirus fiber knob is sufficient to allow a relative degree of escape from preexisting NAb. If confirmed in trials, this approach might improve the efficacy of re-administration of adenoviral gene therapy to humans.

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Introduction

Adenoviral cancer gene therapy has yielded some promising recent clinical results.^{1–3} Heretofore, patient safety has been excellent in cancer trials. Furthermore, even early generation, relatively attenuated agents have provided single-agent tumor responses^{4,5} or improved response rates in randomized combination regimens.^{2,3} Nevertheless, the efficacy of systemic delivery has been disappointing.⁵ Although there are several obstacles to intravenous delivery,⁶ one reason may be the strong immunogenicity of the virus.⁷ As practically all adult humans have been exposed to the most widely used serotype 5 adenovirus (Ad5), the immune system is primed to rapidly produce neutralizing antibodies (NABs) on re-exposure. A high NAB titer may not be limiting for local injection, but it can compromise

systemic delivery.⁸ Other factors that may influence *in vivo* transduction include cellular factors and serum components.

Most patients who die of cancer have disseminated disease and therefore systemic delivery would be attractive. Assuming that bioavailability issues can be resolved,^{9,10} avoiding NAB-mediated opsonization might become a key issue for realization of effective intravenous re-administration. Given the complex nature of advanced human solid tumors, it may be unrealistic to assume that cures can be obtained without multiple rounds of delivery. Therefore, avoiding NAB emerges as a key issue in the development of adenoviral cancer gene therapy approaches.

The adenoviral capsid consists of multiple immunogenic proteins and NAB can be induced against all of them.^{11,12} Because viral serotypes were classically identified based on a lack of cross-reacting antibodies, it is logical that a different serotype can overcome NAB.¹³ Moreover, it has been shown that even small changes in the Ad5 fiber knob can allow escape from preexisting capsid-specific NAB.^{14–17} Although further serological studies are definitely needed, it seems that full neutralization requires NAB against all major capsid proteins, such as hexon, penton and fiber, and there may be

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synergism between them.^{11,12} However, given the key role of the fiber in mediating infection, it is not surprising that anti-fiber NAb seem most important in realizing complete opsonization, and circumventing them can facilitate transduction.^{14–17}

An emerging generation of capsid-modified adenoviruses may be advantageous over Ad5 with regard to gene delivery to human cancer cell lines, clinical tumor specimens and in orthotopic murine models.^{4,6,9,16–22} With regard to non-small-cell lung cancer (NSCLC), the most important cause of cancer mortality globally, our previous work suggests that capsid-modified adenoviruses can yield improved gene delivery and antitumor efficacy in comparison with viruses with the serotype 5 capsid.²³ In particular, modification of the adenovirus capsid with the serotype 3 knob, arginine–glycine–aspartic acid (RGD) in the fiber HI-loop or fiber C-terminal polylysine (pK7) can be useful. However, our previous studies were performed *in vitro* and in immune-deficient animals, and therefore the potential impact of NAb was not accounted for.

Here, we tested changing the adenovirus fiber knob for effective gene delivery in the context of low or high NAb titer, in mice with advanced orthotopic NSCLC and in human clinical samples of both cancerous and non-malignant lung tissue. As expected, the development of NAb toward Ad5-restricted gene delivery with Ad5. Importantly, anti-Ad5 NAb could be overcome with capsid-modified viruses and vice versa.

Results

The effect of NAbs on gene transfer efficacy

NAbs against Ad5(GL), Ad5/3luc1, Ad5lucRGD and Ad5pK7(GL) were induced in immune-competent ICR mice, and their inhibitory effect on Ad5(GL) gene transfer efficacy was analyzed (Table 1). Modifications of the fiber knob are illustrated in a Figure 1. A single subcutaneous injection of Ad5(GL) (a luciferase and green fluorescent protein-expressing virus with an Ad5 capsid) resulted in a 1:4 NAb titer, whereas triple immunization produced a NAb titer capable of complete blocking of *in vitro* gene transfer (Table 2). A single exposure to adenovirus with a different capsid did not induce a measurable anti-Ad5(GL) NAb titer, and even after triple immunization with Ad5/3Luc1 or Ad5pK7(GL), Ad5(GL) was not blocked. NAbs against Ad5lucRGD marginally inhibited Ad5(GL) gene transfer.

The effect of NAbs on gene delivery in immune-competent mice

Immunized mice were injected intravenously with Ad5(GL) and organs were harvested for luciferase measurement (Table 1). A single immunization with Ad5(GL) reduced subsequent gene transfer with Ad5(GL) to the liver ($P < 0.001$), heart ($P < 0.001$), spleen ($P < 0.05$), pancreas ($P < 0.05$) and kidney ($P < 0.01$) (Figure 2). In contrast, single immunization with other viruses did not statistically significantly inhibit Ad5(GL) gene transfer in most organs. We assume that the slight increase in Ad5(GL) gene transfer to lung tissue after immunization with Ad5lucRGD and Ad5pK7(GL) was not due to any possible reason, although a biological effect cannot be excluded.

Triple immunization produced a stronger antibody response, and NAbs against capsid-modified viruses started to affect Ad5(GL). Inhibition of gene transfer was seen in all organs studied except in the heart, where only Ad5(GL) NAbs inhibited Ad5(GL) gene transfer (Figure 3). In contrast to single immunization, where approximately 10-fold reduction in Ad5(GL) gene transfer was seen for most organs, triple immunization reduced liver transduction more than 1000-fold. Gene delivery to the spleen and pancreas were also strongly inhibited. Although a degree of cross-blocking was seen for all viruses, Ad5/3Luc1 (whose fiber knob is from Ad3 and therefore the fiber is least similar to Ad5) was affected

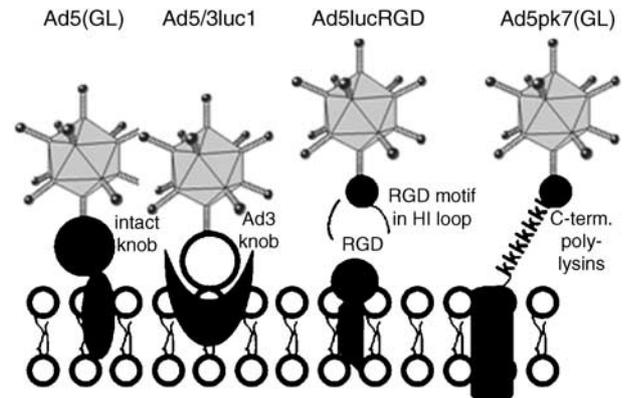


Figure 1 Illustration of fiber modifications of viruses used. Ad5(GL) has normal Ad5 knob, whereas Ad5/3luc1 has knob from Ad3. Ad5lucRGD has arginine–glycine–aspartic acid (RGD) motif incorporated into HI loop of the fiber and pK7(GL) has seven polylysins added into C terminus.

Table 1 Induction of neutralizing antibodies and determination of their effect on gene transfer in immune-competent mice

Group	I	II	III	IV	V	VI	VII	VIII	IX	X
Day										
0 (s.c.)	Ad5(GL)	Ad5(GL)	Ad5/3luc1	Ad5/3luc1	Ad5lucRGD	Ad5lucRGD	Ad5pK7(GL)	Ad5pK7(GL)	Mock	Mock
3 (s.c.)		Ad5(GL)		Ad5/3luc1		Ad5lucRGD		Ad5pK7(GL)		Mock
6 (s.c.)		Ad5(GL)		Ad5/3luc1		Ad5lucRGD		Ad5pK7(GL)		Mock
27	NAb	NAb	NAb	NAb	NAb	NAb	NAb	NAb	NAb	NAb
28 (i.v.)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)	Ad5(GL)
29	Organs and serum	Organs and serum	Organs	Organs	Organs	Organs	Organs	Organs	Organs	Organs

Abbreviations: i.v., intravenous injection (3×10^{10} VP); NAb, determination of neutralizing antibody titer from serum; s.c., subcutaneous immunization (3×10^8 VP); VP, viral particle.

Table 2 Generation of anti-NAbs after subcutaneous injection of adenoviruses

Virus used in immunization	Capsid modification	Ad5 neutralizing titers=1/(dilution blocking>50% of Ad5(GL) gene transfer)	
		Single 3×10^8 VP immunization	Triple 3×10^8 VP immunization
Ad5(GL)	Intact serotype 5 capsid ²⁰	4	>16 384
Ad5/3luc1	Serotype 3 knob in the Ad5 fiber ²⁴	<1	<1
Ad5lucRGD	Arginine-glycine-aspartic acid in the fiber HI-loop ¹⁶	<1	1
Ad5pK7(GL)	Seven lysines in the fiber c-terminus ²⁰	<1	<1

Abbreviations: NAb, Ad5 neutralizing antibody); VP, viral particle.

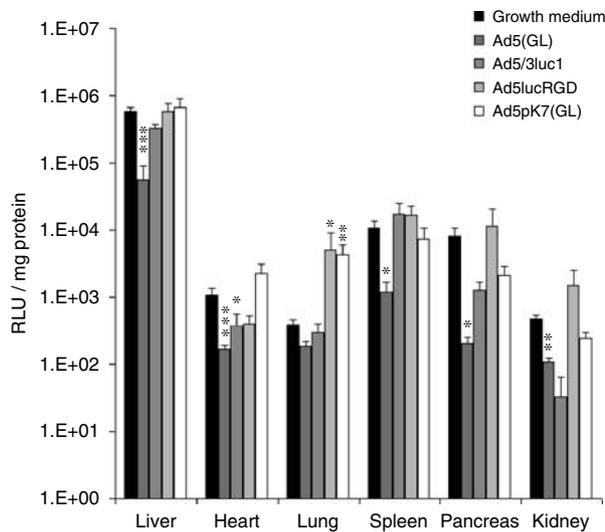


Figure 2 The effect on neutralizing antibodies on biodistribution of Ad5(GL) in immune-competent mice. Mice were immunized subcutaneously with 3×10^8 virus particles (VP) of Ad5(GL), Ad5/3luc1, Ad5lucRGD or Ad5pK7(GL) on day 0. Control animals were immunized with growth medium only. On day 28, 2×10^{10} VP Ad5(GL) was injected intravenously and organs were collected 48 h later for luciferase measurement. Values are mean \pm s.e.m. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by *post-hoc* Dunnett's pairwise multiple comparison *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus mock immunization. Four to six mice were used in each virus group.

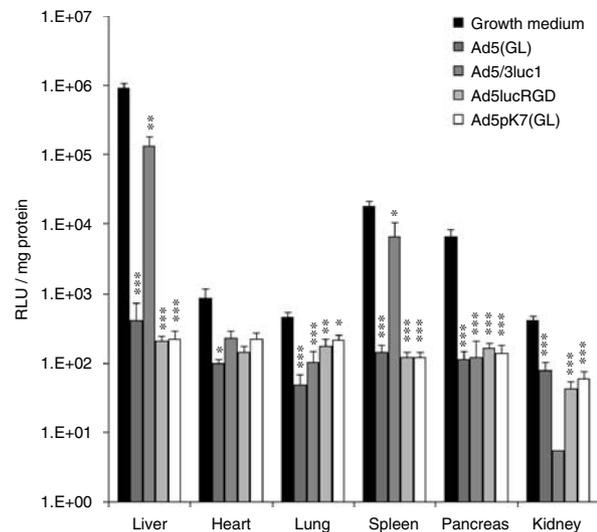


Figure 3 The effect on neutralizing antibodies on biodistribution of Ad5(GL) in immune-competent mice. Mice were immunized subcutaneously with Ad5(GL), Ad5/3luc1, Ad5lucRGD or Ad5pK7(GL) (3×10^8 VP) three times on days 0, 3 and 6. Control animals were immunized with growth medium only. On day 28, 2×10^{10} VP Ad5(GL) was injected intravenously and organs were collected 48 h later for luciferase measurement. Values are mean \pm s.e.m. Statistical analysis was performed by One-way analysis of variance (ANOVA), followed by *post-hoc* Dunnett's pairwise multiple comparison *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus mock immunization. Four to six mice were used in each virus group (except for Ad5/3luc1/kidney, where $N = 1$).

least. Cross-blocking between viruses was seen more clearly *in vivo* than *in vitro*. Partially this may be due to differences in experimental readout (linear versus 50% cutoff), but other factors may play a role.

The effect of NAbs on gene transfer in tumor-bearing mice

Mice bearing advanced orthotopic human lung tumors were injected intravenously with Ad5(GL), Ad5/3luc1, Ad5lucRGD, or Ad5pK7(GL) or with the same viruses preincubated with serum collected from Ad5(GL)-immunized mice. Because of the anti-Ad5 NAb present in that serum, Ad5(GL)-mediated gene transfer into tumors was blocked ($P < 0.01$) (Figure 4a). In contrast, effective gene delivery by all of the capsid-modified viruses was retained. Gene transfer to spleen or liver was not affected

by NAb in this experiment, except at high NAb titers (Supplementary Figure 1).

In the presence of anti-Ad5 antibodies at a 1:512 dilution, 130-, 9- and 35-fold gene transfers were obtained with Ad5/3luc1 ($P = 0.12$), Ad5lucRGD ($P = 0.75$), and Ad5pK7(GL) ($P = 0.038$), respectively (Figure 4b). Modeling a situation with a higher serum NAb titer (1:4 serum dilution), 360-, 3- and 407-fold gene transfers were obtained with Ad5/3luc1 ($P = 0.88$), Ad5lucRGD ($P = 1.00$) and Ad5pK7(GL) ($P = 0.15$), respectively.

Effect of NAbs on gene transfer to human primary NSCLC and normal lung tissue

Two human squamous cell carcinomas (Figures 5, 6a and c), one adenocarcinoma (Figures 5, and 6b) and one

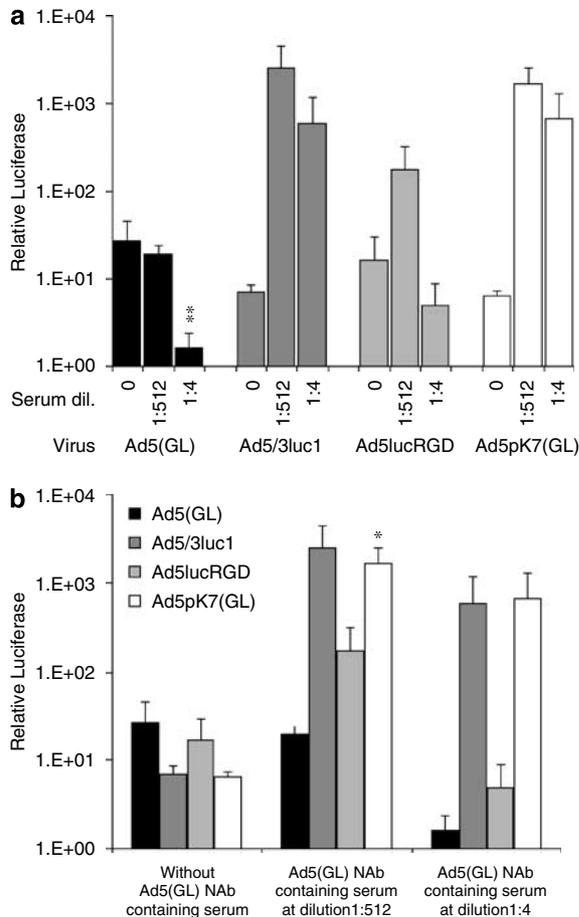


Figure 4 The effect of anti-Ad5(GL) neutralizing antibodies (NABs) on gene transfer in orthotopic tumor-bearing mice. NMRI nude mice were injected into left lung with with 2×10^6 LNM35/EGFP (enhanced green fluorescent proteins) human lung cancer cells. After 9 days, mice were injected intravenously with Ad5(GL), Ad5/3luc1, Ad5lucRGD or Ad5pK7(GL) at 2×10^{10} VP, or with viruses mixed with diluted serum collected from immunocompetent mice immunized with Ad5(GL). After 48 h, tumors ($N = 4$ per group) were harvested and luciferase assay was performed. Data are normalized for total protein content (to allow for possible differences in tumor size) and presented relative to gene transfer obtained with no NAb. Values are mean \pm s.e.m. The same data are displayed in two ways to ease comparison of effect of NABs (a) or performance of viruses (b). Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by *post-hoc* Dunnett's pairwise multiple comparison *t*-test. (a) $**P < 0.01$ virus only versus the corresponding serum containing group. (b) $*P < 0.05$ Ad5(GL) versus Ad5pK7(GL) at serum dilution 1:512. 0 indicates no NAb containing serum added.

non-malignant lung tissue specimen were obtained fresh from patients infected with virus preincubated with serum collected from Ad5(GL) immunized mice. Gene delivery by different viruses was compared in the presence of different dilutions of anti-Ad5(GL) NAb containing serum. Ad5/3luc1 was 3.7- and 190-fold more effective than Ad5(GL) at serum dilutions 1:512 and 1:4, respectively (both $P < 0.001$) (Figure 5a), but degree of cross-blocking was seen in other two samples (Figures 5b and c) ($P < 0.001$). Gene transfer by Ad5pK7(GL) was 10- and 180- fold (Figure 5a) 10- and 20-fold (Figure 5b) at dilutions 1:512 and 1:4, respectively, and 5-fold at dilution 1:512 (Figure 5c) more effective than Ad5(GL) (all $P < 0.001$).

Gene transfer by Ad5(GL) to tumor tissue was decreased by 80% with Ad5(GL) NAb containing serum at a dilution of 1:512 ($P < 0.05$) and completely blocked with serum dilution of 1:4 ($P < 0.05$) (Figure 6a). In other two samples, gene transfer by Ad5(GL) was blocked 85% ($P < 0.001$) at serum dilution of 1:4 (Figures 6b and c). Serum from Ad5(GL)-immunized mice blocked Ad5/3luc1 and Ad5pK7(GL) gene transfer to tumor tissue to lesser degree. Anti-Ad5(GL) NAB decreased Ad5/3luc1 gene transfer by 33% ($P < 0.05$) and 55% ($P < 0.05$) with dilutions of 1:512 and 1:4, respectively (Figure 6a). In Figures 6b and c, the gene transfer of Ad5/3luc1 was decreased by 90 and 85% with dilution of 1:4 ($P < 0.05$). Gene transfer of Ad5pK7(GL) was slightly increased with the 1:512 dilution ($P < 0.05$), but a 69% reduction ($P < 0.05$) in gene transfer was seen at a serum dilution of 1:4 (Figure 6a). Furthermore, 3 and 35% ($P < 0.05$) reduction was seen at dilution of 1:512, and 60 and 90% ($P < 0.05$) reduction at dilution of 1:4 in Figure 6b and c.

In normal lung tissue, anti-Ad5 NAb blocked gene transfer of Ad5(GL) and capsid-modified viruses to similar degree (Figures 5d and 6d). Specifically, Ad5(GL) was blocked 66 and 94%, Ad5/3luc1 90 and 94%, and Ad5pK7(GL) was blocked 74% and 95% with serum dilutions of 1:512 and 1:4, respectively (all $P < 0.05$).

Discussion

Effective re-administration might be an important factor for the realization of the full potential of adenoviral gene therapy for treatment of many diseases. Here, we evaluated the utility of a panel of similar adenoviruses with different fiber knobs for overcoming NAb. Despite no changes in penton, hexon or fiber shaft, we saw effective escape from preexisting NAb induced against a different capsid configuration (Table 2). We used single immunization to model a low level of NAb and triple immunization to simulate a high NAb titer. Triple immunization with Ad5(GL) resulted in high neutralizing activity, which completely blocked *in vitro* gene transfer by Ad5(GL) (Table 2). In contrast, anti-Ad5-lucRGD NAb blocked Ad5(GL) only marginally and anti-Ad5/3luc1 or anti-Ad5pK7(GL) did not block Ad5(GL) at all. These results suggested that even small changes in the fiber knob can allow escape from NAb. These data are in accord and complement previous reports, suggesting that RGD or 5/3 modification of the Ad5 fiber can avoid preexisting anti-Ad5 NAb present in human serum or ascites.^{14–17,25} However, previous studies had not investigated whether NAb induced *in vivo* by various capsid-modified viruses could block Ad5 or vice versa. Also, the absence of cross-blocking between fiber C-terminally modified adenovirus and Ad5 has not been demonstrated before.

To evaluate the significance of NAb *in vivo*, immune-competent mice with NAb against Ad5(GL), Ad5-lucRGD, Ad5/3luc1 or Ad5pK7(GL) were injected intravenously with Ad5(GL), and gene transfer to organs was evaluated (Figure 2). To avoid confounding due to non-identical infectivity, we chose to use a panel of capsid-modified viruses for immunization and just one type of virus for intravenous injection instead of the opposite. As expected, based on the *in vitro* NAb assays

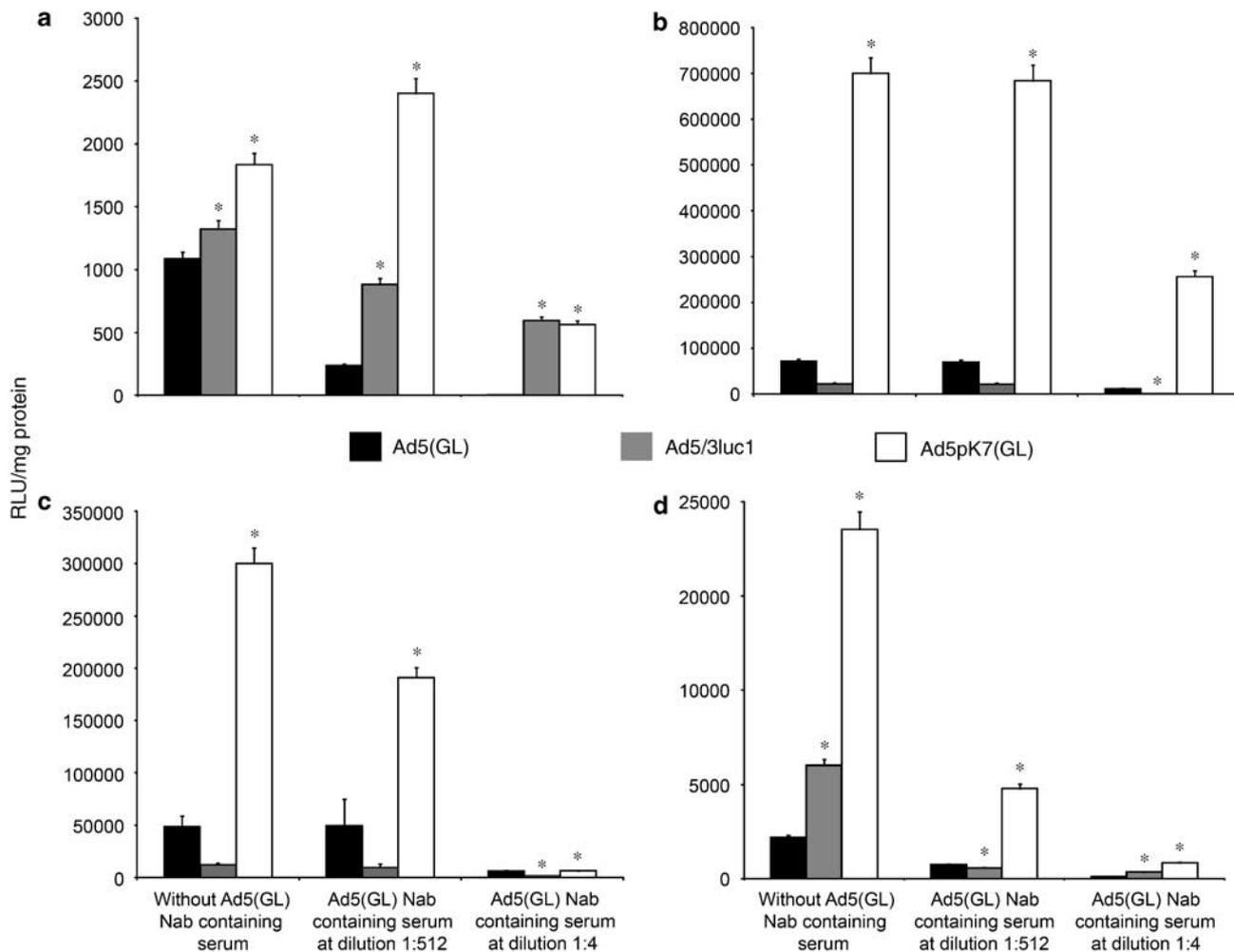


Figure 5 The effect of neutralizing antibodies (NABs) on gene transfer to human primary NSCLC and normal lung tissue. Human lung cancer and non-cancer D tissue explants were infected with Ad5(GL), Ad5/3luc1 and Ad5lucRGD (1000 VP per cell) or viruses preincubated with diluted serum obtained from Ad5(GL) immunized immune-competent mice. After 24 h, luciferase assay was performed. Values are mean \pm s.e.m. The data are grouped by NAb dilution to facilitate comparison of efficacy of viruses in the presence of anti-Ad5 NAb. For statistical analysis, Mann–Whitney test was used. * $P < 0.05$ versus mock immunization.

(Table 2), previous exposure to Ad5(GL) reduced Ad5(GL)-mediated gene transfer to all the main organs. When a higher NAb titer was induced with a triple immunization, a similar but even more pronounced effect was seen (Figure 3). Interestingly, the higher NAb titer revealed cross-blocking activity between capsid-modified viruses and Ad5(GL). NAb generated by triple exposure to Ad5(GL), Ad5lucRGD, Ad5/3luc1 or Ad5pK7(GL) was able to significantly block intravenous Ad5(GL). This suggests that although anti-fiber knob NAb may be most relevant in the context of low NAb titers, very high levels of anti-hexon, anti-penton and anti-fiber shaft NABs can block Ad5(GL) even in the absence of anti-fiber knob NAb. Nevertheless, as Ad5/3luc1 was blocked least, and its fiber knob differs very much from Ad5, anti-fiber knob NAB seem most relevant.

This may be explained by the crucial role of the knob in mediating infection. If binding to the primary receptor cannot occur owing to the action of anti-knob NAb, infection may be thwarted. In contrast, if primary binding can occur due to the absence of anti-knob NAb, infection may be able to proceed despite NAB

against other capsid proteins. Although for Ad5 it is known that the penton base–integrin interaction is important, this may not be the case for Ad5lucRGD, Ad5/3luc1 or Ad5pK7(GL), whose entry may be possible without a secondary interaction. These aspects are difficult to study because the Ad3 receptor is still unknown, Ad5lucRGD binds to a large number of $\alpha_v\beta$ integrins and cannot be blocked with recombinant integrins, and penton base RGD-mutant versions of Ad5pK7(GL) have not been published.²⁶ However, the existence of human and non-human adenovirus serotypes that lack RGD in penton base, but can infect human cells, supports the notion of entry without the requirement for penton–integrin interactions.^{27,28} Further studies are needed to dissect the putative role of fiber shaft in the context of infection in the presence of NAB.^{29–31} Nevertheless, the empiric assays performed here suggest that anti-fiber knob NABs have an important role in determining the success of gene transfer *in vitro* and *in vivo*.

To study how NABs affect systemic gene transfer to orthotopic lung cancer, human lung cancer xenografts were established in the left lung of mice. This model is

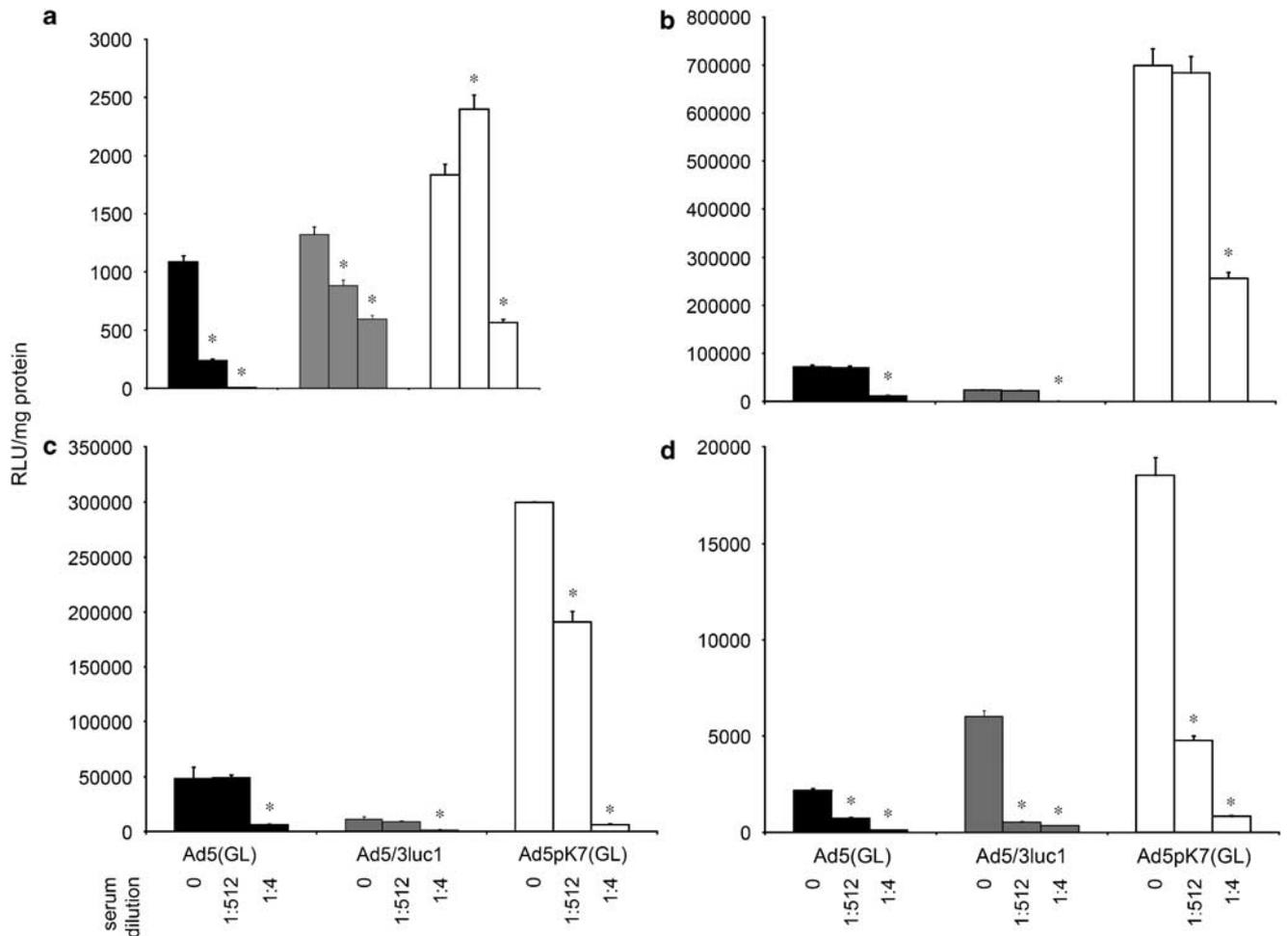


Figure 6 The effect of neutralizing antibodies (NABs) on gene transfer to human primary NSCLC and normal lung tissue. Human lung cancer and non-cancer D tissue explants were infected with Ad5(GL), Ad5/3luc1 and Ad5lucRGD (1000 VP per cell) or viruses preincubated with diluted serum obtained from Ad5(GL) immunized immune-competent mice. After 24 h, luciferase assay was performed. The same data as shown in Figure 5 are now grouped to allow evaluation of the effect of anti-Ad5 NAb on fiber-modified viruses. Values are mean \pm s.e.m. For statistical analysis, the Mann–Whitney test was used. * $P < 0.05$ versus mock immunization.

aggressive and the treatment is refractory, and therefore it resembles most human NSCLC cases.²³ A low titer of anti-Ad5 NAb did not block intravenous Ad5(GL), but a high titer almost completely prevented gene transfer (Figure 4a). Regardless of anti-Ad5 NAb titer, effective gene delivery was achieved with Ad5lucRGD, Ad5/3luc1 and Ad5pK7(GL). When the same data were evaluated from a different perspective, it was found that Ad5lucRGD, Ad5/3luc1 and Ad5pK7(GL) were able to deliver genes to orthotopic lung tumors more effectively than Ad5(GL) in both the low and high NAb scenarios (Figure 4b). These data suggest that changing the fiber knob can retain or increase gene delivery to orthotopic lung tumors when anti-Ad5 NABs are present. Furthermore, there was no evidence that intravenous gene delivery to orthotopic tumors by Ad5lucRGD, Ad5/3luc1 or Ad5pK7(GL) would be compromised by anti-Ad5 NAB. However, when interpreting these results, it should be taken into account that NAB cannot be induced in mice with human tumors, because human xenografts can only be grown in immune-deficient animals, and therefore the *in vivo* data discussed here provide only indirect evidence.

To extend these data into human clinical specimens of both normal human lung and NSCLC, explants fresh from patients were infected in the presence or absence of anti-Ad5 NAb (Figures 5 and 6). Ad5(GL) was completely blocked by a high NAb titer, whereas Ad5/3luc1 and Ad5pK7(GL) continued to allow gene transfer. Both Ad5/3luc1 and Ad5pK7(GL) were more effective than Ad5(GL) in gene delivery in the presence of low or high NAB. Intriguingly, anti-Ad5 NAB blocked gene transfer by capsid-modified viruses to normal human lung explants much more than what was seen with tumor explants. This suggests that fiber-knob-independent (but perhaps fiber shaft dependent) mechanisms that were postulated as important in the context of systemic biodistribution in mice and primates^{29–31} may also apply to humans. Experiments in syngeneic mouse or hamster models might shed more light on these phenomena.

To our knowledge, these data are the first in assessing the effect of NAB on adenoviral gene transfer to human normal and cancerous lung. Further experiments should evaluate whether the knob is more relevant with regard to tumor transduction, whereas normal tissue transduc-

tion is more dependent on the shaft. If confirmed, this might mean that switching the fiber knob to avoid NAb can retain gene transfer to the tumor while not affecting NAb-mediated protection of normal tissues, which could increase the therapeutic window of the treatment. Theoretically, these differences might be caused by the differences in vasculature. Leaky and immature tumor vessels³² might allow viruses to escape vessels more easily than in normal tissues where the endothelium is intact. Intriguingly, the shaft has been proposed to bind to heparan sulfate proteoglycans,^{29–31} which are abundant in endothelia.³³ Blocking this interaction with anti-shaft NAb (the shaft is identical in the viruses used here) might reduce normal tissue gene transfer, whereas tumor tissue transduction could still occur through knob-mediated mechanisms.

In summary, our data suggest that switching the fiber knob of adenovirus can allow effective systemic re-administration despite NAb. Nevertheless, only relative escape is achieved, because cross-blocking is seen with high NAb titers. As Ad5/3luc1 is most different from Ad5(GL) and was blocked least by anti-Ad5 NAb, it might be interesting to study viruses even further distinct from Ad5. For example, using viruses pseudotyped with the entire fiber (instead of just the knob) or hexon might yield further advances.^{34,35} A hexon-switch experiment would help understand whether anti-fiber NABs are unique with regard to sero-switching for efficacy, or whether the same might be true for any anti-capsid NAB.³⁶

Moreover, completely non-Ad5 serotypes might be useful,^{13,28,37,38} if the appealing attributes of Ad5 (for example, high gene expression, *in vivo* stability, easy production, safety) can be retained. This should not be taken for granted, as Ad5 has become the single most popular gene therapy vector because of these attributes, and there is no guarantee that they would apply to other serotypes. Obviously, it would be interesting to confirm these preclinical results in humans. However, given the daunting task of performing a clinical trial even with just one virus in the current translational environment, it seems ambitious to attempt to obtain sufficient funding for the production of multiple viruses for a phase 1 trial.

Materials and methods

Cell lines and viruses

A549 lung adenocarcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA USA), and LNM35/EGFP (enhanced green fluorescent proteins) human large cell lung carcinoma cells were obtained from Dr Takashi Takahashi (Institute of Medical Science, Advanced Clinical Research Center City, Tokyo, Japan). The main features of the viruses used in this study are described in Table 2. All viruses were E1-deleted, propagated on 293 cells and purified on cesium chloride gradients. The viral particle (VP) concentration was determined at 260 nm, and standard plaque assay on 293 cells was performed to determine infectious particles. The ratios of VP/infectious particles were 21, 11, 23, and 10 for Ad5(GL), Ad5/3luc1, Ad5lucRGD and Ad5pK7(GL), respectively.

Animals

Athymic 3- to 4-week-old NMRI nude and immune-competent ICR mice (Taconic, Ejby, Denmark) were quarantined for 2 weeks before experiments were started. Mice were housed in plastic cages with wire mesh covers in a room with a 12-h light–dark cycle with $50 \pm 10\%$ relative humidity at $24 \pm 1^\circ\text{C}$ (nude mice) or at $22 \pm 1^\circ\text{C}$ (ICR mice). All animal experiments were approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland.

Induction of NABs

ICR mice were randomized into 10 groups of 6 mice each and immunized with viruses subcutaneously (Table 1). Ad5(GL), Ad5/3luc1, Ad5lucRGD or Ad5pK7(GL) were injected either once on day 0 or thrice on days 0, 3 and 6. The dose was 3×10^8 VP in a 100 μl volume of Opti-MEM (contains 0% serum (FCS) and 1% antibiotics and glutamine). Control animals were mock immunized with Opti-MEM only. On day 27, blood was collected from the hindlimb artery, allowed to clot and serum was separated by centrifugation and stored in -20°C for further experiments.

Assessment of NAB titers

A total of 1×10^4 A549 cells per well were plated on 96-well plates and cultured overnight. Serum was incubated at 65°C for 60 min to inactivate complement, and a fourfold dilution series of serum was prepared in Opti-MEM. Ad5(GL) (100 VP per cell) was mixed with diluted serum samples and incubated at room temperature for 30 min and the mix was added to cells in quadruplicates. Two hours later, the infection media was replaced by fresh growth media. After 48 h, luciferase expression was measured by FluoStar Optima luminometer (BMG LabTech, Offenburg, Germany) according to the manufacturer's instructions. To evaluate the effect of NAB in the serum of mice immunized with adenoviruses, luciferase values normalized for the effect of serum alone were plotted relative to gene transfer achieved with the highest dilution (1:16 384). A trend line was calculated to control for variation, and the NAB titer was determined as the lowest degree of dilution that blocked gene transfer more than 50%.

The effect of NABs on biodistribution in immune-competent ICR mice

Immunized ICR mice were injected intravenously with 3×10^{10} VP of Ad5(GL), and 48 h later, organs (liver, heart, lung, pancreas, spleen and kidney) were harvested and frozen in -80°C . Organs were homogenized in cell culture lysis buffer (Promega, Madison, WI, USA), and luciferase expression was measured by FluoStar Optima luminometer. The blood from the mice immunized with Ad5(GL) was collected by intracardiac aspiration and serum was separated. Background luciferase values (organs of mice not injected with virus) were subtracted.

The effect of NABs on gene transfer in tumor-bearing mice

The left lung of NMRI nude mice was injected with 2×10^6 LNM35/EGFP cells in a total volume of 200 μl using a syringe with a 27-gauge needle.²³ Before

injection, the mice were anesthetized with Medetomidine+Ketalar (1:2) (Domitor, Orion Pharma, Espoo, Finland, Ketalar, Pfizer, New York, NY, USA). Tumors were allowed to grow for 9 days. Mice were randomized into 12 groups ($N=4$ per group) and injected intravenously with 2×10^{10} VP of Ad5(GL), Ad5/3luc1, Ad5-lucRGD and Ad5pK7(GL), or with the same viruses mixed with heat-inactivated serum diluted at 1:512 or 1:4. This serum was pooled from ICR mice immunized once or thrice with Ad5(GL). Tumors were harvested 48 h later and luciferase assay was performed by FluoStar Optima. The data are protein normalized (to account for possible differences in tumor size) and presented relative to gene transfer obtained with no NAb.

The effect of NAb on gene transfer to human primary NSCLC and normal lung tissue

Human NSCLC and normal lung tissue samples obtained with informed consent were homogenized²² and infected with Ad5(GL), Ad5/3luc1 and Ad5lucRGD (1000 VP per cell). Viruses were preincubated with (0, 1:512 and 1:4 dilutions) inactivated serum from Ad5(GL)-immunized mice at room temperature for 30 min before infection of tissue explants. A luciferase assay was performed in quadruplicate 24 h later by the Luciferase Assay System (Promega, Madison, WI, USA). Tissue availability did not allow analysis of all viruses.

Statistical analyses

Analyses were performed using SPSS 14.0 Software for Windows, and comparisons between treatment groups were carried out using one-way analysis of variance (ANOVA), followed by Dunnett's pairwise multiple comparison *t*-test. A *P*-value less than 0.05 was considered statistically significant. For clinical samples, the Mann-Whitney test was used.

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Supplementary Information accompanies the paper on Gene Therapy website (<http://www.nature.com/gt>)