multiplicity of infection (MOI) representing the number of viral particles added per target cell, with approximately 1000 viral particles equivalent to 10 plaque forming unit (pfu) for the Ad/GFP vector. Vectors expressing GFP (Ad/GFP and Ad-RGD/GFP) were employed to assess transduction efficiency, whereas vectors expressing OVA (Ad/OVA and Ad-RGD/OVA) were used to assess the impact of viral transduction on the activation of OVA-specific T cells.

2.4. Transduction of DC with Ad vectors

DC were transduced with Ad vectors as described previously (Harui et al., 2004). Briefly, 2.5 to 5.0×10^5 DC were exposed to a suspension of Ad at different MOI (25 to 250 virus particles per target cell) in 200 μl of medium containing 100 μl PBS and 100 μl RPMI-1640 supplemented with 2% FBS. Co-cultures were incubated for 2 h at 37 °C in either a humidified incubator or in a temperature-regulated centrifuge at 2000×*g* (Centrifuge 5415R/Eppendorf, Westbury, NY) according to the protocol described by Nishimura et al. (2001a). An additional 800 μl of complete media containing GM-CSF and IL-4 (20 ng/ml of each) were then added and cultures incubated for another 48 h before analysis.

2.5. Blocking Ad transduction with exogenous RGD-peptide

In selected experiments, BM-DC (5×10^5) were pre-incubated (4 °C, 60 min) with either 20 uM or 200 uM RGD-4C peptide (AnaSpec, San Jose, CA) in order to block $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Huang et al., 1995). Ad vectors were then added (MOI 1000) and co-cultures incubation with/without centrifugation (2000×*g*) at 4 °C for the first 60 min and 37 °C for the second 60 min. Cells were treated with trypsin-EDTA (0.25 mg/ml–1 mM, Invitrogen, Calsbad, CA) for 2 min to eliminate vectors that had not already been internalized and with DNAse (Sigma) for 10 min (0.02 mg/ml, 37 °C) to degrade extracellular DNA. Cells were washed and incubated at 37 °C for 48 h with GM-CSF and IL4 (20 ng/ml of each) prior to analysis.

2.6. Measurement of transgene expression and DC phenotype

48 h after transduction, DC were identified by their expression of CD11c (Caltag Laboratories, Burlingame, CA) and their concurrent expression of α_v integrin (CD51, BD Pharmingen) and/or GFP measured by flow cytometry using a FACScalibur cytometer (Becton Dickinson, San Jose, CA) and FCS Express analysis software (De Novo Software, Ontario, Canada). Cells were gated for $CD11c^+$ DC cells and assessed for the percentage of DC that expressed GFP and for the mean fluorescent intensity (MFI) of the transduced subset. The percentage of cells transduced was multiplied by the MFI to obtain an index of total GFP expression. Expression of DC markers on the CD11c population was also assessed using a panel of biotinylated antibodies against MHC I, MHC II, B7.1 and B7.2 in combination with a secondary streptavidin-APC (all from Caltag Laboratories).

2.7. Antigen specific T cell proliferation assay

The capacity for transduced DC to activate transgene-specific T cells was assessed by co-culturing with T cells from OT-1 transgenic mice. DC transduced with either Ad/OVA or Ad-RGD/OVA at MOI 25 and 250, that had been subjected to either centrifugation (2000×*g*) or standard culture, were harvested 48 h after transduction. T cells were isolated from lymph nodes of OT-1 mice and the $CD8^+$ subset purified by negative depletion using anti-B220, anti-Gr.1, anti-NK1.1 and anti-CD4 mAb and complement. Assays were performed in round-bottomed 96-well culture plate in 200 μl RPMI 1640 containing 10% FCS and 10^{-4} M 2-ME. OVA-specific $CD8^+$ T cells (2×10^5) were co-cultured with transduced DC at DC:T ratios of 1:25, 1:50, and 1:100 for 3 days, then pulsed with 1.25 uCi (46.2 kBq) [H^3]-thymidine and thymidine incorporation determined 12 h later by liquid scintillation counting.

2.8. Statistical analysis

Data from FACS analyses are represented as the percentage of $CD11c^+$ DC (minimum of 5000 total events) expressing fluorescence for a given marker at levels above that of unstained controls and the MFI of the positive population. Proliferation of OT-1 cells is expressed as the mean value±S.D. of triplicate cultures with significant differences between groups determined by a 2-tailed Student's *t*-test when p were ≤ 0.05.

3. Results

3.1. Ad-RGD tranduce DC more efficiently than conventional Ad

Mouse BM-DC were transduced by either capsid-modified Ad-RGD/GFP or the conventional Ad/GFP to

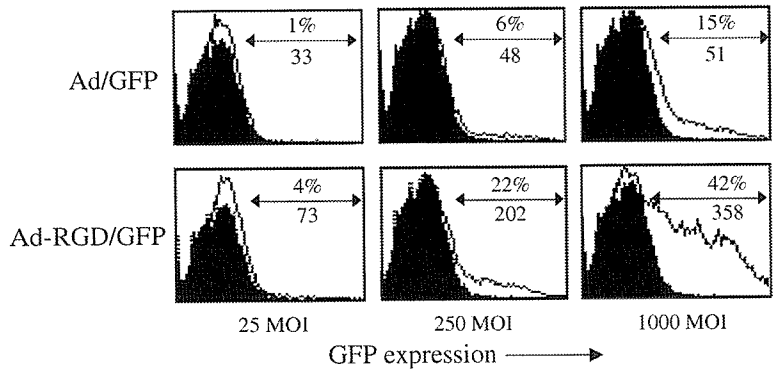


Fig. 1. Transgene expression in DC is enhanced by Ad-RGD/GFP. Mouse BM-DC were harvested after 7–9 days of culture and transduced in vitro in 200 μl of medium (2% FCS) with either Ad/GFP or Ad-RGD/GFP vectors at MOI of 25, 250 or 1000. After 2 h, cells were resuspended in RPMI-160 medium with 10% FCS, GM-CSF and IL-4 (20 ng/ml of each). GFP expression by $CD11c^+$ DC was determined 48 h later by flow cytometry with the percentage of cells expressing GFP and the MFI of the positive population shown for each condition. Representative experiment shown, $n=3$.

evaluate their transduction efficiency at various MOI (25, 250 and 1000). Transgene expression was detected after 48 h by FACS (Fig. 1) Transduction of DC by Ad/GFP resulted in 1±0.1%, 6±0.5% and 15±1.0% of the cells being transduced at the different MOI, respectively. In contrast, transduction employing the Ad-RGD/GFP vector resulted in transduction rates of 4±1.17%, 22±0.3% and 42±2.6% at the same MOI. The expression of GFP (MFI) was also significantly higher in DC exposed to Ad-RGD/GFP (73, 202 and 358 at the different MOI)

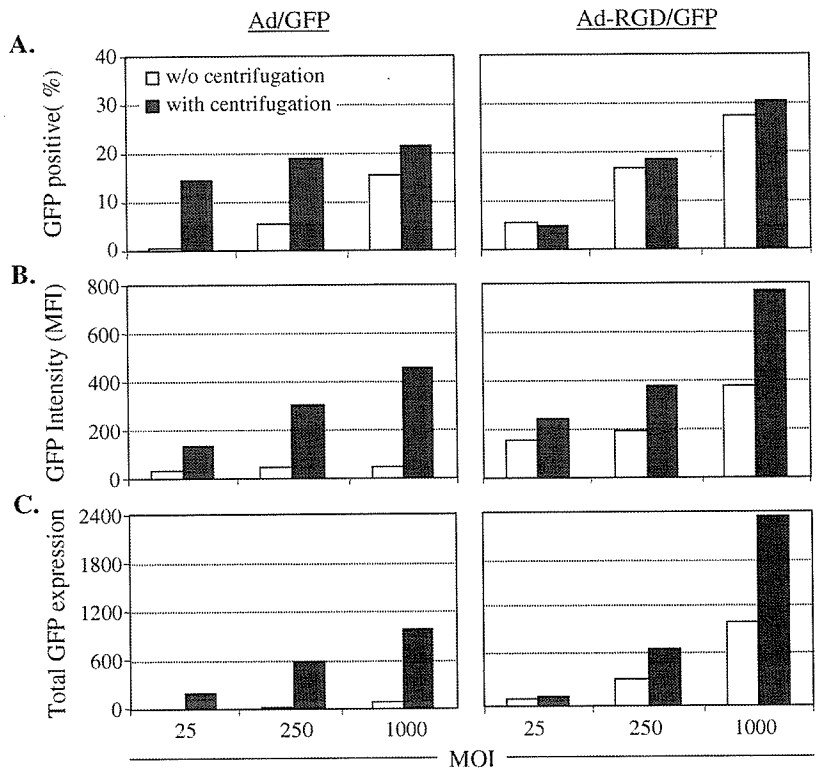


Fig. 2. Enhancement of Ad transduction by centrifugal force. Mouse BM-DC were transduced at different MOI (25, 250 or 1000) with either Ad-GFP or Ad-RGD-GFP, in the presence or absence of centrifugal force (2000×g), for 2 h at 37 °C. Cells were then cultured for 48 h and GFP expression measured by flow cytometry. A) Percentage of $CD11c^+$ DC expressing GFP. B) MFI of GFP expression by transduced DC. C) Total GFP expression (% transduced DC × MFI) by DC population. Representative experiment, $n=3$.

as compared to cells exposed to Ad/GFP. These results indicate that an Ad-RGD vector can be used to significantly enhance transgene expression by Ad-exposed DC.

3.2. Centrifugation increases transduction efficiency and transgene expression by DC

We also evaluated the use of centrifugal force as an approach for enhancing the transduction of DC (Nishimura et al., 2001a,b). Centrifugation at 2000×*g* increased both the percentage of DC transduced and the level of transgene expression, and effects were observed to some degree with both the Ad/GFP and Ad-RGD/GFP vectors (Fig. 2). At MOI 25–1000, centrifugation with Ad/GFP increased transduction efficiency by 1.4- to 2-fold and resulted in 14.7±0.3%, 19.0±2.9% and 21.6±2.5% of DC expressing GFP (Fig. 2A). The combination of centrifugation with the Ad-RGD/GFP vector resulted in only a ~1.1-fold increase in the percentage of transduced DC (Fig. 2A). While the effect on transduction efficiency was only significant when centrifugation was combined with Ad/GFP, the GFP expression level (MFI) significantly increased when centrifugation was employed with either vector. Centrifugation enhanced GFP expression by 4- to 77-fold when employed with Ad/GFP and 2- to −9-fold when combined with Ad-RGD/GFP (Fig. 2B). When transduction rate and GFP expression were multiplied to give an overall index of GFP expression, total GFP expression was enhanced 12- to 88-fold by combining centrifugation with Ad/GFP and 1.4- to 2.3-fold by combining centrifugation with Ad-RGD/GFP (Fig. 2). Despite the small impact on GFP expression by the Ad-RGD, the combination of Ad-RGD/GFP with centrifugation still provided the most efficient approach for loading DC with the transgene antigen.

3.3. RGD peptide inhibits Ad-mediated transduction

The finding that centrifugation dramatically increased the transduction frequency when combined with the conventional Ad/GFP vector, but had only modest effects when combined with the modified Ad-RGD/GFP vector suggested that both approaches might be competing for the same binding sites. To evaluate whether centrifugation enhanced transduction by increasing Ad binding to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins, we added exogenous RGD synthetic peptide (RGD-4C) as a specific and competitive inhibitor of this pathway. When 20 uM and 200 uM of RGD peptides were added to the medium

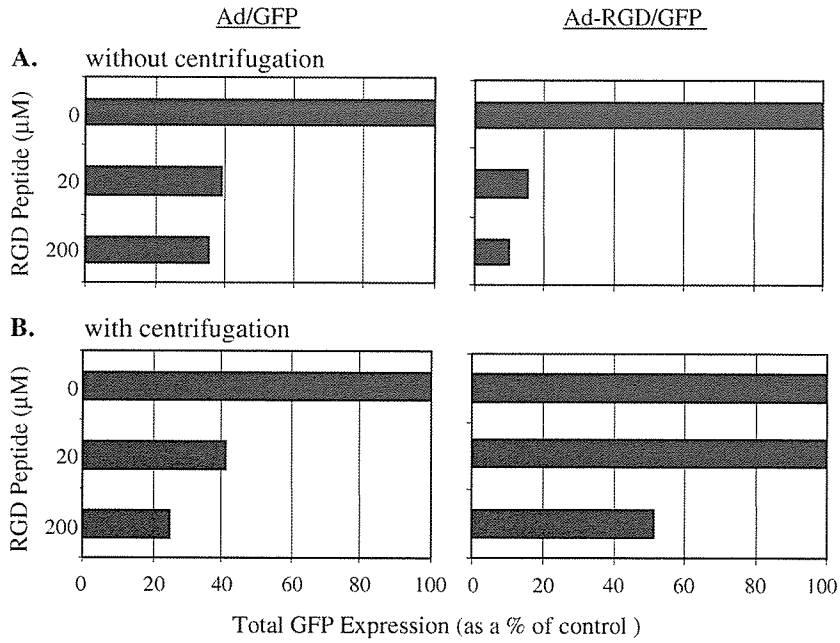


Fig. 3. RGD peptide blocks transduction. Mouse BM-DC were incubated with the RGD-4C peptide (20 uM and 200 uM) at 4 °C for 1 h prior to transduction with Ad/GFP or Ad-RGD/GFP (MOI 1000) for 2 h in the presence or absence of centrifugal force (2000×*g*). GFP expression was determined by flow cytometry 48 h later. A) Total GFP expression (% transduced DC × MFI) produced by Ad-GFP and Ad-RGD/GFP presented as a percentage of control expression in the absence of blocking peptide. B) Total GFP expression (% transduced DC × MFI) produced by Ad-GFP and Ad-RGD/GFP when combined with centrifugation and presented as a percentage of control expression in the absence of blocking peptide. Representative experiment, $n=3$.

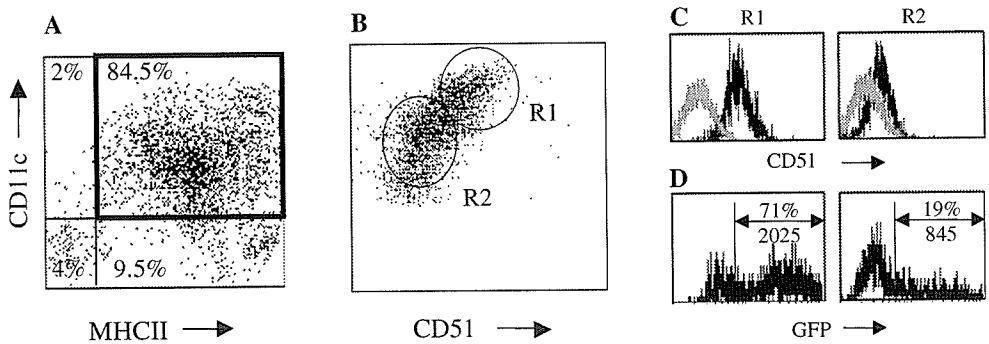


Fig. 4. DC transduction is restricted primarily to the subset of cells expressing high levels of CD51. Cells from the bone marrow-derived cultures were harvested after 7 days GM-CSF and IL-4 and transduced (2 h, 37 °C) with Ad-RGD/GFP (MOI 1000) in the presence of centrifugal force (2000×g), followed by culture for an additional 48 h. A) Within the bone marrow-derived cultures, 80–90% of the cells were typical BM-DC as defined by the concurrent expression of both CD11c and MHC II. B) BM-DC, defined by their expression of CD11c, were examined for their expression of CD51 by flow cytometry. C) Cells were then gated into two populations, one expressing high levels of CD51 (R1) and one expressing low levels of CD51 (R2). D) GFP expression was then independently analyzed on the CD51 high (R1) and CD51 low (R2) DC subsets as a measure of the susceptibility of these two subpopulations to transduction by Ad-RGD/GFP. Representative experiment, $n=8$.

prior to Ad infection, total GFP expression was reduced by 60.8% and 64.4% in the setting of transduction with Ad/GFP alone, and by 84.7% and 89.7% in cultures containing Ad-RGD/GFP, respectively (Fig. 3A). Similarly, when transduction was performed in the setting of centrifugation, addition of RGD peptides at the same

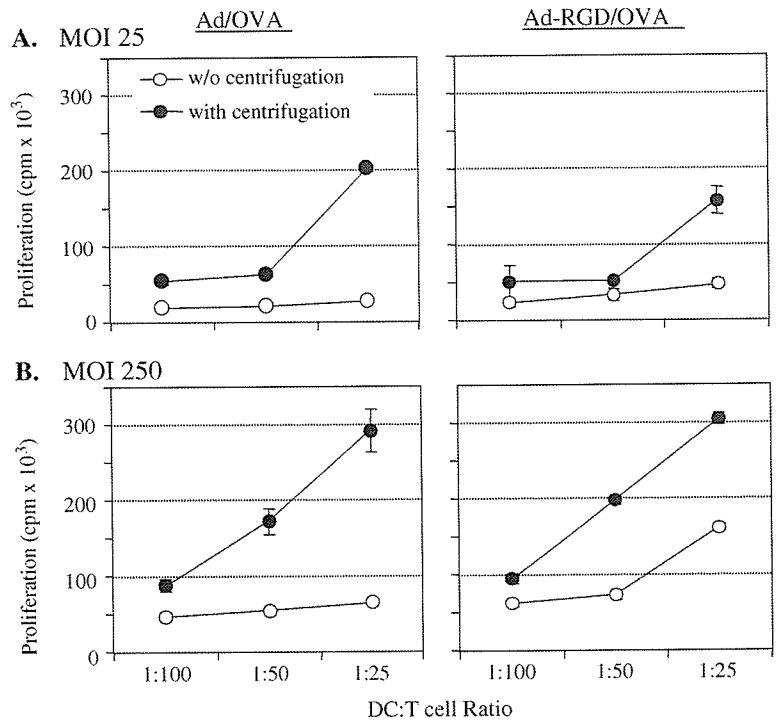


Fig. 5. The capacity for Ad-transduced DC to stimulate transgene-specific T cells depends upon the type of vector, the MOI and the presence or absence of centrifugation. BM-DC were harvested after 7 days of culture and transduced at a MOI of either 25 (A) or 250 (B) with either Ad/OVA or Ad-RGD/OVA in the presence or absence of centrifugal force (2000×g). DC were harvested 48 h later and co-cultured for 3 days at multiple DC:T cell ratios (1:25–1:100) with 2×10^5 OVA specific $CD8^+$ T cells purified from the lymph nodes of OT-1 mice. Cultures were pulsed with 1.25 uCi (46.2 kBq) [H^3]-thymidine and cells harvested 12 h later for determination of proliferation by liquid scintillation counting. Results are the mean±S.E. of triplicate cultures. Representative experiment, $n=3$.

concentrations resulted in a decrease in GFP expression by 58.6% and 75.1% with Ad/GFP and by 0.5% and 48.00% with Ad-RGD/GFP, respectively (Fig. 3B). As such, RGD peptides competitively inhibited transduction by both of these vectors alone, as well as the enhancement observed when combined with centrifugation, suggesting common mechanisms of action.

3.4. The enhanced transduction resulting from the use of Ad-RGD and/or centrifugation occurred primarily in DC expressing high levels of α_v integrins

We next used a FACS-based analysis to determine the characteristics of the cells that were transduced by the RGD-Ad vector alone or in combination with centrifugation. Out of the entire bone marrow-derived culture, 80–90% of the cells expressed CD11c and MHC II, identifying them as typical BM-DC (Fig. 4). Only 22–30% of these $CD11c^+$ BM-DC expressed high levels of α_v ($CD51^{bright}$) (Fig. 4, R1), whereas 68–70% of BM-DC expressed only low levels of α_v ($CD51^{dim}$) (Fig. 4, R2). Within these two subsets, defined by their bright or dim expression of CD51, GFP expression (MFI) was 2025±27.8 within $CD51^{bright}$ DC but only 845±31 within $CD51^{dim}$ DC, again suggesting that transduction by Ad-RGD alone, or in combination with centrifugation, works primarily via an integrin-dependent pathway. Consistent with this, the effects of RGD blocking peptide were also primarily on the $CD51^{bright}$ DC subset (data not shown).

3.5. Expression of MHC and activation markers on transduced DC

The expression of activation markers MHCI, MHCII, B7.1 and B7.2 on DC transduced with Ad/GFP or Ad-RGD/GFP, under standard conditions or in conjunction with centrifugation, was analyzed by FACS. There were no remarkable changes in the expression of these DC activation/maturation markers, although a slight and isolated increase in the expression of MHC I was observed when DC were transduced with Ad-RGD/GFP. Centrifugation did not enhance the expression of any of these markers.

3.6. Use of an Ad-RGD vector alone, and/or with centrifugation, significantly enhances the capacity for DC to activate transgene specific T cells

Ad vectors encoding OVA were used to evaluate the functional consequences that these different modes of transduction had on the capacity for DC to stimulate OVA-specific T cells obtained from OT-1 transgenic mice. DC were transduced with Ad/OVA or Ad-RGD/OVA at MOI 25 (Fig. 5A) or MOI 250 (Fig. 5B), with/without centrifugation, cultured 48 h and then added to wells containing $CD8^+$ OT-1 cells. The magnitude of T cell stimulation was dose dependent with respect to both vectors. DC transduced at MOI 250 induced ~6-fold higher levels of T cell proliferation then did DC transduced at MOI 25 when the Ad-RGD/OVA vector was used and 2–4-fold higher levels of proliferation were noted in the setting of the Ad/OVA vector. However, a significant enhancement was also detected when DC transduced with vectors alone were compared to DC transduced in combination with centrifugation. T cell activation was also more pronounced when DC were transduced with Ad-RGD/OVA as compared to Ad/OVA, and the enhancement due to centrifugation was most prominent when combined with the Ad/OVA vector as compared to the Ad-RGD/OVA vector (Fig. 5A,B).

3.7. Transgene expression correlates with antigen specifice T cell stimulation

Finally, in order to study the inter-relationship between transgene expression and transgene-specific T

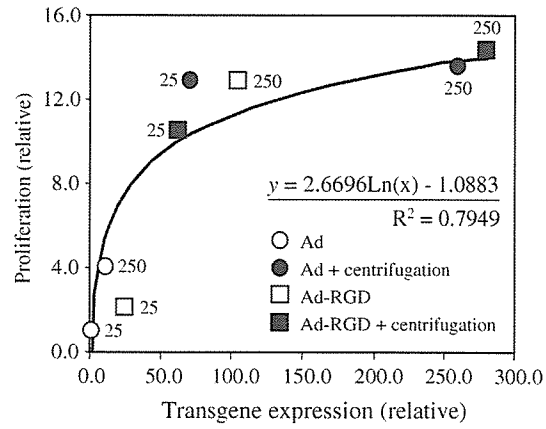


Fig. 6. The expression of transgene antigens by DC correlates with their capacity to activate transgene-specific T cells. BM-DC were transduced with Ad/GFP or Ad-RGD/GFP (MOI 25 or 250 as indicated in the figure), in the presence or absence of centrifugation, and total GFP expression (% transduced DC × MFI) determined relative to the subset of DC transduced with Ad/GFP MOI 25 alone. Similarly, BM-DC were transduced with Ad/OVA or Ad-RGD/OVA (MOI 25 or 250 as indicated in the figure), in the presence or absence of centrifugation, and their capacity to stimulate the proliferation of OT-1 cells (DC/T cell ratio of 1:50) determined relative to the subset of DC transduced with Ad/OVA MOI 25 alone. Data was pooled from two sets of gene expression assays and two sets of T cell stimulation assays and the mean values evaluated for a correlation between transgene expression and T cell proliferation by a logarithmic regression analysis [$y = \ln(x) + b$].

cell activation, we pooled results from two sets of assays in which DC were either transduced with GFP-expressing vectors to measure antigen loading or the same DC were transduced with OVA-expressing vectors to measure T cell activation (Fig. 6). All results within a given assay were normalized so that the activity produced by the conventional Ad/GFP vector, in the absence of centrifugation, was assigned a value of 1. The enhancement observed by either increasing the MOI, by employing an Ad-RGD vector, or by combining with centrifugation, was calculated as a ratio of the measured activity to that produced by the Ad/GFP vector alone. A direct comparison between the impact of these techniques on antigen expression and on T cell activation yielded a highly significant logarithmic relationship (R^2=0.795; Fig. 6).

4. Discussion

The magnitude of antigen loading is an essential factor in determining the capacity for DC to activate immune responses (Bronte et al., 1997). Ad-based gene therapy has provided an important approach for enhancing the antigen-presenting activity of DC and for targeting their subsequent stimulatory activity against defined tumor antigens (Basak et al., 2002; Basak et al., 2004; Reichardt and Brossart, 2004; Jeras et al., 2005; Wu et al., 2005). However, DC lack expression of CAR, the primary Ad receptor (Okada et al., 2003). To address this obstacle, a number of Ad vectors with modified capsids, such as an Ad5/Ad35 chimera vector or fiber-modified Ad-RGD vectors have been designed (Okada et al., 2001; Slager et al., 2004). With respect to the Ad-RGD vector, it was hypothesized that placing an additional RGD binding sequence on the fiber knob would enhance the capacity for viral particles to bind DC. The RGD motif is normally expressed only on the penton base, where it is stericly hindered in its ability to interact with the $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrins expressed on target cells (Reynolds et al., 1999; Mizuguchi et al., 2001). As such, expression of an additional RGD motif on the fiber knob not only increases the number of binding sites but the direct interaction with cell surface integrins.

Since the α_v integrin molecule (CD51) dimerizes with both β_3 and β_5, CD51 expression was used as an overall measure of RGD binding receptors (Brossart et al., 1997; Okada et al., 2003; Varnavski et al., 2003). Tagging CD51 with a fluorescent labeled monoclonal antibody also allowed us to differentiate BM-DC into two populations, one $CD51^{bright}$ and the other $CD51^{dim}$. Finally, the availability of soluble RGD peptides allowed us to block this cell surface receptor and evaluate its role in the transduction process. Using these approaches, we examined both Ad-RGD vectors and centrifugation for the mechanisms by which they enhance DC transduction. By employing matching Ad and Ad-RGD vectors that expressed GFP we were able to directly measure both the frequency of Ad transduction and the level of transgene expression (Fig. 1). Compared to Ad/GFP, infection with Ad-RGD/GFP resulted in a 9 to 20-fold increase in total transgene expression, effects which were observed regardless of the MOI examined. The Ad-RGD/GFP vector increased both the transduction efficiency (by 3- to 4-fold) as well as the magnitude of transgene expression (by 2 to 7-fold). As previously suggested, we hypothesized that this enhancement was directly related to the capacity for this vector to bind integrins expressed on the surface of the DC.

It has been reported that the addition of centrifugal force (centrifugation) during transduction increases viral entry when human DC (Nishimura et al., 2001a,b), monocytes or macrophages (Mayne et al., 2003) are employed as targets. Little is known about the exact mechanisms by which centrifugation works and we hypothesized that it might have an additive or even synergistic effect when combined with the Ad-RGD vector. Indeed, we found that centrifugation increased overall transgene expression by DC when combined with either conventional or Ad-RGD vectors (Fig. 2). However, the magnitude and pattern of the enhancement was not the same in each case. Combining centrifugation with the Ad/GFP vector dramatically increased both the number of transduced cells and their level of expression, reaching maximal values very similar to that obtained using the Ad-RGD vector alone. In contrast, combining centrifugation with Ad-RGD/GFP had almost no effect on the number of transduced cells and only a modest effect on the level of transgene expression. We interpreted these results to indicate that both of these approaches, placing an extra RGD motif on the fiber knob and adding centrifugal force, might be acting through the same pathway—increasing the interaction of RGD motifs with cell surface integrin receptors.

Several approaches were used to evaluate this hypothesis. RGD peptides inhibited both baseline levels of transgene expression produced by the Ad and Ad-RGD vectors, as well as their augmentation by centrifugation (Fig. 3). Interestingly, the effects produced by soluble RGD peptides were not as dramatic when used to block Ad-RGD transduction in the setting of centrifugation. This might be interpreted in two

different manners. First, the effects were still dose dependent and it is possible that higher concentrations of RGD peptide would have lead to complete blockade. This would suggest that the combination of centrifugal force and the expression of RGD sequences on the fiber knob result in a significant increase in the avidity of their interaction with surface integrins. Alternatively, if RGD peptides fail to completely block transduction in this setting, it would suggest that a component of viral entry is integrin independent.

To address this question by another approach, we next analyzed transgene expression by the $CD51^{bright}$ and $CD51^{dim}$ subpopulations of DC. As shown in Fig. 4, when $CD11c^{+}$ DC cells were transduced by the Ad-RGD/GFP vector in combination with centrifugation, GFP expression by $CD51^{bright}$ DC was ~5 times higher than that by the $CD51^{dim}$ population, suggesting that the enhancement in gene expression under these circumstances was primarily α_v integrin-specific. Similar result was observed when this approach was used to examine transduction by the conventional AdV alone, or when combined with centrifugation (data not shown).

It has also been reported that Ad-RGD mediated transduction leads to activation of mouse DC resulting in enhanced MHC class and co-stimulatory expression (Hirschowitz et al., 2000; Korst et al., 2002; Salucci et al., 2005). To compare the activation status of DC transduced with conventional Ad and Ad-RGD, in the presence or absence of centrifugation (MOI 1000), we examined their expression of several activation/maturation markers—MHCI, MHC II, B7.1 and B7.2 by FACS. No dramatic changes in the expression of these molecules were detected. Results similar to ours have been reported by others (Nishimura et al., 2001a,b), suggesting that other factors or technical features, other than the transduction process itself, may account for the activation that others have reported.

As antigen presenting cells, DC play an important roll in the activation of antigen specific T cells and the induction of cellular immunity. Therefore, by employing OVA-expressing vectors, we examined these different transduction techniques for their effects on T cell activation. DC transduced with the Ad-RGD/OVA vector induced superior activation of OVA specific T cells compared to the results observed after using the Ad-OVA vector (Fig. 5). In an analogous manner, the addition of centrifugation further increased the magnitude of antigen specific T cell responses. However, the relationship between the effects on antigen loading and T cell activation was logarithmic, with T cell activation reaching a plateau with only minor increases in proliferation despite further increases in antigen expression. These findings suggest that T cell activation becomes relatively antigen dose independent after an optimal level of antigen exposure (Miller et al., 2003; Herrera et al., 2002; Okada et al., 2001). In this respect it is interesting to note that increasing the viral load of a conventional Ad vector by 10-fold (MOI 25 versus 250) had only modest effects on increasing T cell stimulation and was far from optimal. However, optimal T cell activation was reached by either combining centrifugation with a conventional Ad vector or by increasing the dose of the Ad-RGD vector alone. While combining the Ad-RGD vector with centrifugation significantly increased antigen loading, this combination had relatively little impact on boosting T cell activation. Thus, we propose that for optimizing antigen presentation in vitro, the use of an RGD vector or the combination of a conventional Ad vector with centrifugation might produce similar results. However, when considering the direct administration of these vectors in vivo the RGD-Ad vector may have a significant advantage (Worgall et al., 2004).

In conclusion, our results demonstrated that Ad vectors with an RGD-modified viral capsid targets cell surface integrin receptors and efficiently transduce DC to produce high levels of transgene expression. The addition of centrifugation during the transduction process can further enhance antigen loading. The concentration-dependent inhibition of transduction by soluble RGD peptides and the selective transduction of $CD51^{bright}$ target cells that occurs when either Ad-RGD vectors alone or in combination with centrifugation are employed, strongly suggest that both of these approaches work by increasing interaction with cell surface integrins. Finally, there appears to be a logarithmic relationship between antigen loading and the capacity for transduced DC to activate T cell responses. Simply increasing the dose of a conventional Ad vector has a relatively minor impact on T cell activation when compared to either combining this approach with centrifugation, or replacing the Ad vector with an Ad-RGD vector. Either of these approaches might be used to optimize in vitro loading of DC, while the use of an Ad-RGD vector may have unique advantages when considering their application in vivo.

Acknowledgements

This work was supported by the Tobacco-Related Disease Research Program of California (SKB, 10KT-0086); the American Lung Association of California (SKB) and the Cancer Research Program, California Department of Health Services (MDR, #TTP1019), Human Gene medicine seed Grant UCLA (SKB). The

Jonsson Comprehensive Cancer Center/UCLA provided core facilities for flow cytometry and cell sorting.

References

Adams, S., O'Neill, D.W., Bhardwaj, N., 2005. Recent advances in dendritic cell biology. J. Clin. Immunol. 25 (3), 177.

Arthur, J.F., Butterfield, L.H., Roth, M.D., Bui, L.A., Kiertscher, S.M., Lau, R., Dubinett, S., Glaspy, J., McBride, W.H., Economou, J.S., 1997. A comparison of gene transfer methods in human dendritic cells. Cancer Gene Ther. 4 (1), 17.

Basak, S.K., Harui, A., Stolina, M., Sharma, S., Mitani, K., Dubinett, S.M., Roth, M.D., 2002. Increased dendritic cell number and function following continuous in vivo infusion of granulocyte macrophage-colony-stimulating factor and interleukin-4. Blood 99, 2869.

Basak, S.K., Kiertscher, S., Harui, A., Roth, M.D., 2004. Modifying adenoviral vectors for use as gene-based cancer vaccines. Viral Immunol. 17 (2), 182.

Belousova, N., Korokhov, N., Krendelshchikova, V., Simonenko, V., Mikheeva, G., Triozzi, P.L., Aldrich, W.A., Banerjee, P.T., Gillies, S.D., Curiel, D.T., Krasnykh, V., 2003. Genetically targeted adenovirus vector directed to CD40-expressing cells. J. Virol. 77 (21), 11367.

Bronte, V., Carroll, M.W., Goletz, T.J., Wang, M., Overwijk, W.W., Marincola, F., Rosenberg, S.A., Moss, B., Restifo, N.P., 1997. Antigen expression by dendritic cells correlates with the therapeutic effectiveness of a model recombinant poxvirus tumor vaccine. Proc. Natl. Acad. Sci. U. S. A. 94, 3183.

Brossart, P., Goldrath, A.W., Butz, E.A., Martin, S., Bevan, M.J., 1997. Virus-mediated delivery of antigenic epitopes into dendritic cells as a means to induce CTL. J. Immunol. 158 (7), 3270.

Gunzer, M., Grabbe, S., 2001. Dendritic cells in cancer immunotherapy. Crit. Rev. Immunol. 21, 133.

Harui, A., Roth, M.D., Kiertscher, S.M., Mitani, K., Basak, S.K., 2004. Vaccination with helper-dependent adenovirus enhances the generation of transgene-specific CTL. Gene Ther. 11 (22), 1617.

Herrera, O.B., Brett, S., Lechler, R.I., 2002. Infection of mouse bone marrow-derived dendritic cells with recombinant adenovirus vectors leads to presentation of encoded antigen by both MHC class I and class II molecules—potential benefits in vaccine design. Vaccine 21 (3–4), 231.

Hirschowitz, E.A., Weaver, J.D., Hidalgo, G.E., Doherty, D.E., 2000. Murine dendritic cells infected with adenovirus vectors show signs of activation. Gene Ther. 7 (13), 1112.

Huang, S., Endo, R.I., Nemerow, G.R., 1995. Upregulation of integrins alpha v beta 3 and alpha v beta 5 on human monocytes and T lymphocytes facilitates adenovirus-mediated gene delivery. J. Virol. 69 (4), 2257.

Jenne, L., Schuler, G., Steinkasserer, A., 2001. Viral vectors for dendritic cell-based immunotherapy. Trends Immunol. 22 (2), 102.

Jeras, M., Bergant, M., Repnik, U., 2005. In vitro preparation and functional assessment of human monocyte-derived dendritic cells —potential antigen-specific modulators of in vivo immune responses. Transpl. Immunol. 14 (3–4), 231.

Korst, R.J., Mahtabifard, A., Yamada, R., Crystal, R.G., 2002. Effect of adenovirus gene transfer vectors on the immunologic functions of mouse dendritic cells. Molec. Ther. 5 (3), 307.

Mayne, G.C., Borowicz, R.A., Greeneklee, K.V., Finlay-Jones, J.J., Williams, K.A., Hart, P.H., 2003. Centrifugation facilitates transduction of green fluorescent protein in human monocytes and macrophages by adenovirus at low multiplicity of infection. J. Immunol. Methods 278 (1–2), 45.

Miller, G., Lahrs, S., Shah, A.B., DeMatteo, R.P., 2003. Optimization of dendritic cell maturation and gene transfer by recombinant adenovirus. Cancer Immunol. Immunother. 52 (6), 347.

Mittereder, N., March, K.L., Trapnell, B.C., 1996. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J. Virol. 70 (11), 7498.

Mizuguchi, H., Koizumi, N., Hosono, T., Utoguchi, N., Watanabe, Y., Kay, M.A., Hayakawa, T., 2001. A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. Gene Ther. 8 (9), 730.

Nakamura, M., Iwahashi, M., Nakamori, M., Ueda, K., Ojima, T., Naka, T., Ishida, K., Yamaue, H., 2005. Dendritic cells transduced with tumor-associated antigen gene elicit potent therapeutic antitumor immunity: comparison with immunodominant peptide-pulsed DCs. Oncology 68 (2–3), 163.

Nemerow, G.R., Stewart, P.L., 1999. Role of alpha(v) integrins in adenovirus cell entry and gene delivery. Microbiol. Mol. Biol. Rev. 63 (3), 725 (Sep.).

Nishimura, N., Nishioka, Y., Shinohara, T., Sone, S., 2001a. Enhanced efficiency by centrifugal manipulation of adenovirus-mediated interleukin 12 gene transduction into human monocyte-derived dendritic cells. Hum. Gene Ther. 1;12 (4), 333.

Nishimura, N., Nishioka, Y., Shinohara, T., Ogawa, H., Yamamoto, S., Tani, K., Sone, S., 2001b. Novel centrifugal method for simple and highly efficient adenovirus-mediated green fluorescence protein gene transduction into human monocyte-derived dendritic cells. J. Immunol. Methods 1;253 (1–2), 113.

Okada, N., Saito, T., Masunaga, Y., Tsukada, Y., Nakagawa, S., Mizuguchi, H., Mori, K., Okada, Y., Fujita, T., Hayakawa, T., Mayumi, T., Yamamoto, A., 2001. Efficient antigen gene transduction using Arg–Gly–Asp fiber-mutant adenovirus vectors can potentiate antitumor vaccine efficacy and maturation of murine dendritic cells. Cancer Res. 61 (21), 7913.

Okada, N., Masunaga, Y., Okada, Y., Iiyama, S., Mori, N., Tsuda, T., Matsubara, A., Mizuguchi, H., Hayakawa, T., Fujita, T., Yamamoto, A., 2003. Gene transduction efficiency and maturation status in mouse bone marrow-derived dendritic cells infected with conventional or RGD fiber-mutant adenovirus vectors. Cancer Gene Ther. 10 (5), 421.

Reichardt, V.L., Brossart, P., Kanz, L., 2004. Dendritic cells in vaccination therapies of human malignant disease. Blood Rev. 18 (4), 235.

Reynolds, P., Dmitriev, I., Curiel, D., 1999. Insertion of an RGD motif into the HI loop of adenovirus fiber protein alters the distribution of transgene expression of the systemically administered vector. Gene Ther. 6 (7), 1336.

Rossi, M., Young, J.W., 2005. Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. J. Immunol. 175 (3), 1373.

Roth, M.D., Cheng, Q., Harui, A., Basak, S.K., Mitani, K., Low, T.A., Kiertscher, S.M., 2002. Helper-dependent adenoviral vectors efficiently express transgenes in human dendritic cells but still stimulate antiviral immune responses. J. Immunol. 15 169 (8), 4651.

Salucci, V., Lena, A.M., Ciliberto, G., Scarselli, E., La Monica, N., 2005. Adenovirus transduction and culture conditions affect the immunogenicity of murine dendritic cells. Scand. J. Immunol. 62 (3), 206.

Sarukhan, A., Camugli, S., Gjata, B., von Boehmer, H., Danos, O., Jooss, K., 2001. Successful interference with cellular immune responses to immunogenic proteins encoded by recombinant viral vectors. J. Virol. 75 (1), 269.

Slager, E.H., van der Minne, C.E., Goudsmit, J., van Oers, J.M., Kostense, S., Havenga, M.J., Osanto, S., Griffioen, M., 2004. Induction of CAMEL/NY-ESO-ORF2-specific CD8+ T cells upon stimulation with dendritic cells infected with a modified Ad5 vector expressing a chimeric Ad5/35 fiber. Cancer Gene Ther. 11 (3), 227.

Steitz, J., Tormo, D., Schweichel, D., Tuting, T., 2005. Comparison of recombinant adenovirus and synthetic peptide for DC-based melanoma vaccination. Cancer Gene Ther. 13 (3), 318.

Varnavski, A.N., Schlienger, K., Bergelson, J.M., Gao, G.P., Wilson, J.M., 2003. Efficient transduction of human monocyte-derived dendritic cells by chimpanzee-derived adenoviral vector. Hum. Gene Ther. 14 (6), 533.

Verma, I.M., Weitzman, M.D., 2005. Gene therapy: twenty-first century medicine. Annu. Rev. Biochem. 74, 711.

Wickham, T.J., Mathias, P., Cheresh, D.A., Nemerow, G.R., 1993. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 23;73 (2), 309.

Worgall, S., Busch, A., Rivara, M., Bonnyay, D., Leopold, P.L., Merritt, R., Hackett, N.R., Rovelink, P.W., Bruder, J.T., Wickham, T.J., Kovesdi, I., Crystal, R.G., 2004. Modification to the capsid of the adenovirus vector that enhances dendritic cell infection and transgene-specific cellular immune responses. J. Virol. 78 (5), 2572.

Wu, Q., Xia, D., Carlsen, S., Xiang, J., 2005. Adenovirus-mediated transgene-engineered dendritic cell vaccine of cancer. Curr. Gene Ther. 5 (2), 237.

Gene Therapy (2006) 13, 1118–1126

www.nature.com/gt

SHORT COMMUNICATION

Adenovirus serotype 35 vector-mediated transduction into human CD46-transgenic mice

F Sakurai[1], K Kawabata[1], N Koizumi[1], N Inoue[2], M Okabe[2], T Yamaguchi[3], T Hayakawa[4] and H Mizuguchi[1,5]

[1]*Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka, Japan;* [2]*Genome Information Research Center, Osaka University, Osaka, Japan;* [3]*Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, Tokyo, Japan;* [4]*Pharmaceuticals and Medical Devices Agency, Tokyo, Japan and* [5]*Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan*

We previously demonstrated that systemic administration of adenovirus serotype 35 (Ad35) vectors to mice does not mediate efficient transduction in organs, probably because expression of the mouse analog of the subgroup B Ad receptor, human CD46 (membrane cofactor protein), is limited to the testis. Here, we describe the in vitro *and* in vivo *transduction characteristics of Ad35 vectors by using homozygous and hemizygous human CD46-transgenic (CD46TG) mice, which ubiquitously express human CD46. An Ad35 vector more efficiently transduced the primary dendritic cells and macrophages prepared from CD46TG mice than those from wild-type mice.* In vivo *transduction experiments demonstrated that CD46TG mice are more susceptible to Ad35 vector-mediated* in vivo *transduction than are wild-type mice. In particular, homozygous CD46TG mice, which express higher levels of CD46 in the organs than hemizygous CD46TG mice, tend to exhibit higher transduction efficiencies after intraperitoneal administration than hemizygous CD46TG mice. Intraperitoneal administration of Ad35 vectors resulted in efficient transduction into the mesothelial cells of the peritoneal organs in homozygous CD46TG mice. These results indicate that an Ad35 vector recognizes human CD46 as a cellular receptor in CD46TG mice. However, the* in vivo *transduction efficiencies of Ad35 vectors in CD46TG mice are much lower than those of conventional Ad5 vectors in wild-type mice.*
Gene Therapy (2006) **13,** 1118–1126. doi:10.1038/sj.gt.3302749; published online 16 March 2006

Keywords: *adenovirus serotype 35 vector; human CD46; human CD46-transgenic mice; receptor; biodistribution*

The human adenoviruses (Ads) comprise a group of 51 serologically distinct viruses.[1,2] Among them, Ad vectors widely used for gene therapy are based on Ad serotype 5 (Ad5), which belongs to subgroup C. Adenovirus serotype 5 vectors have several attractive features as gene delivery vehicles; for example, they have noteworthy *in vivo* transduction efficiency and transduction ability into both proliferating and non-proliferating cells. In addition, Ad5 vectors can be grown to high titer, and large stretches of foreign DNA can be inserted into the Ad5 vector genome.

However, recent studies have revealed several disadvantages associated with the clinical use of Ad5 vectors. One disadvantage is the high prevalence of adult humans (>50%) who produce neutralizing antibodies to Ad5.[3,4] Pre-existing neutralizing antibodies prevent Ad vectors from transducing cells *in vivo*.[5] Furthermore, Ad vector preimmunization in mice has been demonstrated to significantly increase vector-mediated liver toxicity on re-exposure.[6] Therefore, pre-existing immunity to Ad5 vectors greatly hampers the *in vivo* application of Ad5 vectors. Inefficient transduction with Ad5 vectors of cells lacking expression of a primary receptor for Ad5, coxsackievirus and adenovirus receptor (CAR), is also highly problematic. Several important target cells for gene therapy, including hematopoietic stem cells,[7] dendritic cells (DCs)[8] and malignant tumor cells,[9] express low levels of CAR.

To overcome these drawbacks of Ad5 vectors, several groups (including ours) have developed Ad vectors composed of other human Ad serotypes, such as Ad serotype 7a,[10] 11[4,11] and 35,[12–15] and Ads of animal origin, such as chimpanzee,[16] bovine,[17] mouse[18] and ovine.[19] Among these non-Ad5 vectors, those composed of human Ad11 and Ad35, which belong to subgroup B, are highly promising as gene transfer vectors for the following reasons. First, Ad11 and Ad35 are serotypes least neutralized by serum from healthy human blood donors: less than 20% of serum samples are positive for anti-Ad11 and -Ad35 neutralizing antibodies.[14] Second, human subgroup B Ads, including Ad11 and Ad35, recognize human CD46 (membrane cofactor protein) as a cellular receptor,[20,21] although Ads belonging to subgroups A, C, D, E and F use CAR as a primary receptor. CD46 is a single-chain type I transmembrane glycoprotein that is ubiquitously expressed in all cells (except erythrocytes) in humans, suggesting that human sub-

Correspondence: Dr H Mizuguchi, Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, 7-6-8 Asagi, Saito, Ibaragi-City, Osaka 567-0085, Japan.
E-mail: mizuguch@nibio.go.jp
Received 29 September 2005; revised 19 January 2006; accepted 21 January 2006; published online 16 March 2006

group B Ads can infect almost all human cell types, regardless of CAR expression. We previously demonstrated that Ad35 vectors show broad tropism toward human cells,[12,13,22] including CAR-negative cells, because of the ubiquitous expression of CD46. Furthermore, CD46 expression is highly upregulated in human malignant tumor cells,[23,24] suggesting that these cells would be suitable targets for Ad35 vector-mediated transduction.

Adenovirus serotype 35 vectors exhibit efficient transduction in a variety of human cells *in vitro*. In contrast, systemic administration of Ad35 vectors into mice mediates low levels of transduction efficiencies in organs.[13,14] The refractoriness of mice to Ad35 vectors would be owing to the expression pattern of CD46 in this host. Whereas CD46 is ubiquitously expressed in humans, expression of mouse CD46 is limited to the testes. In addition, mouse and human CD46 s are only 46% similar.[25] These differences indicate that the conventional mouse is not a suitable small animal model for characterization of Ad35 vector-mediated *in vivo* transduction. A small animal model in which the transduction properties of Ad35 vectors can be characterized appropriately is essential to estimate the efficiency of Ad35 vector-mediated transduction in humans.

In the present study, to evaluate the *in vivo* transduction properties of Ad35 vectors in an animal model that ubiquitously expresses CD46 (as do humans), we administered Ad35 vectors intravenously and intraperitoneally into homozygous and hemizygous human CD46-transgenic (CD46TG) mice, which have a human CD46 gene inserted into the mouse genome. Our results indicate that CD46 acts as an attachment receptor for Ad35 after *in vivo* administration, but the transduction efficiencies in organs were lower than we had expected.

First, Western blot analysis was performed to examine human CD46 expression levels in the organs of homozygous and hemizygous CD46TG mice. As shown in Figure 1, human CD46 was ubiquitously expressed in all organs examined of homozygous and hemizygous CD46TG mice. In particular, amounts of CD46 were higher in the liver, spleen, lung and kidney than in other organs. CD46 expression patterns in CD46TG mice mimicked those observed in humans, which were reported previously,[26] and homozygous CD46TG mice expressed CD46 more abundantly than hemizygous mice. CD46 expression in the liver, spleen and diaphragm of homozygous mice was 3.2, 3.7 and 3.2 times that, respectively, in these organs of the hemizygous mice. We also confirmed that human CD46 expression levels in the primary hepatocytes, splenocytes and thymocytes of the homozygous mice were similar to, or lower than, those in human cultured cell lines (human hepatoma and leukemia lines; data not shown). Flow cytometric and Western blotting analysis failed to detect CD46 expression in the erythrocytes of our CD46TG mice (data not shown), although detectable levels of CD46 were expressed in the erythrocytes of some CD46TG mice lines used in other studies.[27,28]

Next, we performed *in vitro* transduction of an Ad35 vector into bone marrow-derived dendritic cells (mBM-DC) and peritoneal macrophages prepared from CD46TG and wild-type mice. Recently, the potential utility of Ad35 vectors as vaccine vectors has been proposed,[29,30] and DC and macrophages are considered

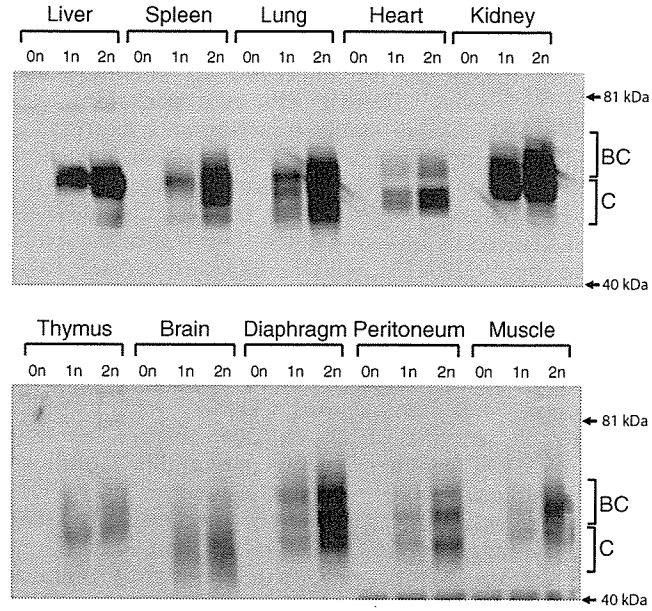


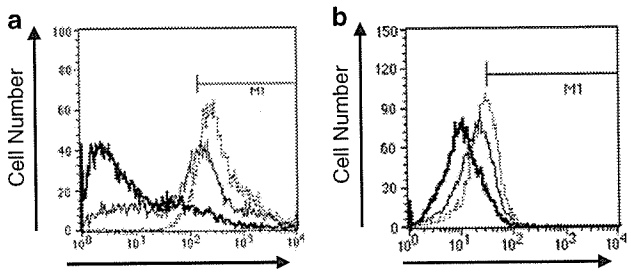
Figure 1 Human CD46 expression in organs harvested from CD46TG mice. The protein samples were prepared from wild-type mice (0n), hemizygous CD46TG mice (1n) and homozygous CD46TG mice (2n). The molecular masses of marker proteins (kDa) and approximate positions of the two major isoforms of the CD46 proteins (BC and C isoforms) are indicated on the right. CD46TG mice were produced as follows. Spermatozoa were dispersed from the epididymis of mature (>12 weeks old) B6D2F1 male mice into 400 μl of TYH medium[50] and were frozen in liquid nitrogen immediately after dilution with TYH medium to 1×10^7/ml. The bacterial artificial chromosome DNA carrying human CD46 (5 μg/ml in TE) (GenomeSystems Inc., St Louis, MO, USA) was added to thawed sperm after purification by using the Large-Construct Kit (Qiagen, Valencia, CA, USA). The mixture was incubated for 5 min at room temperature and then diluted with 9 volumes of 12% PVP-HCZB. Metaphase II oocytes for microinjection were prepared from B6D2F1 female mice, as described previously.[51] These oocytes were maintained in potassium simplex optimized medium (kSOM) under mineral oil equilibrated in 5% (v/v) CO_2 in air at 37°C until use. For microinjection, sperm heads were aspirated into a pipette attached to a piezoelectric pipette-driving unit, and a sperm head was injected into each oocyte, as described previously.[51] After injection, the eggs were incubated in kSOM until two-cell stage, and were transferred to ICR pseudo-pregnant foster mothers. CD46TG mice were detected among the pups born by using genomic PCR as described previously.[40] After backcrossing to the C57Bl6 background for more than five generations, homozygous CD46TG mice were obtained by mating hemizygous mice. Homozygous CD46TG mice were identified by mating CD46TG mice with wild-type mice. For Western blotting analysis, organs collected from wild-type mice (C57Bl6, female, 5 weeks old, obtained from Nippon SLC Co. Ltd, Shizuoka, Japan) and CD46TG mice (female, 5 weeks old) were homogenized in phosphate-buffered saline containing 1% Triton-X, 2 mM EGTA and proteinase inhibitor cocktail (1 mM PMSF, 1 μg/ml pepstatin and 1 μg/ml leupeptin). After centrifugation of the homogenates, the supernatants (7.5 μg protein per sample) underwent nonreducing sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis, and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific binding, CD46 was detected with anti-CD46 rabbit serum (1:5000; kindly provided by Dr T Seya, Hokkaido University, Japan), followed by incubation in the presence of peroxidase-labeled anti-rabbit antibody (1:6000). Signals on the membrane were visualized and analyzed as described previously.[52]

to be ideal targets for immunotherapy using Ad35 vector-based vaccines. Bone marrow-derived dendritic cells[31] and peritoneal macrophages[32] were prepared as

described previously. An Ad35 vector that expresses green fluorescence protein (GFP), Ad35GFP, was prepared by means of an improved *in vitro* ligation method[22,33,34] using 293-E1B cells as a packaging cell line. 293-E1B cells are stable transformants expressing Ad35E1B proteins, which were generated by transfection of pEF-Ad35E1B into 293 cells and after selection with G418 (Invitrogen, Carlsbad, CA, USA). pEF-Ad35E1B was constructed by insertion of the fragment of the Ad35 genome (bp 1911–3413), which contains the Ad35 E1B-55 K gene, into pEF/myc/nuc (Invitrogen). The plaque-forming unit (PFU)-to-particle ratios of Ad35GFP in 293-E1B cells was 1:66.

Flow cytometric analysis showed that mBM-DC from hemizygous and homozygous CD46TG mice express considerable amounts of human CD46 (Figure 2a): 47% of the mBM-DC from hemizygous mice and 87% of those from homozygous mice were CD46-positive (% of M1-gated). Transduction experiments demonstrated that Ad35GFP mediated more efficient transduction in mBM-DC from CD46TG mice than from wild-type mice (Figure 3a). Ad35GFP at a dose of 3000 vector particles (VP)/cell successfully transduced about 42% of the mBM-DC from the hemizygous CD46TG mice and 83% of those from homozygous mice. In contrast, only 3.8% of the mBM-DC from wild-type mice were positive for GFP expression, a rate that is only slightly above background level. In addition, mean fluorescence intensity data revealed that the mBM-DC from CD46TG mice were more susceptible to Ad35 vector than those from wild-type mice. Similar results were obtained for peritoneal macrophages. Human CD46 was expressed in 20% of the peritoneal macrophages from hemizygous CD46TG mice and in 41% of those from homozygous animals (Figure 2b). Infection by Ad35GFP resulted in 10% GFP-positive macrophages from hemizygous CD46TG mice and in 20% GFP-positive macrophages among those from homozygous transgenic mice. In contrast, the macrophages from wild-type mice were refractory to Ad35 vector-mediated transduction (Figure 3b). These results indicate that human CD46 expression greatly increases the transduction efficiency of Ad35 vector in mouse primary cells and that the *in vitro* transduction efficiency of Ad35 vector depends on CD46 expression density.

The refractoriness of mBM-DC and macrophages from wild-type mice also suggests inefficient interaction between the RGD motif in the penton base of Ad35 vector and αv-integrins on the cells. The hypervariable RGD loop in the penton base of the Ad35 vector is supposed to be shorter than that of conventional Ad5 viruses because the RGD loop of Ad11 (55 amino acids), the sequence of which is identical to that of Ad35, is shorter than that of Ad2 (74 amino acids).[35] It suggests that αv-integrins on the cell surface might be less easily accessible to the RGD motif in the penton bases of Ad35 viruses, compared with Ad5 vectors. Conventional Ad5 vectors transduce to varying degrees via interaction



	% of M1-gated
Wild-type	8.2 %
Hemi TG	46.6 %
Homo TG	86.6 %

	% of M1-gated
Wild-type	5.0 %
Hemi TG	19.8 %
Homo TG	40.5 %

Figure 2 CD46 expression in (**a**) bone marrow-derived dendritic cells (mBM-DC) and (**b**) peritoneal macrophages from wild-type mice and CD46TG mice. Thick lines, thin lines and dotted lines represent cells from wild-type mice (C57Bl6, 5 weeks old), and hemizygous (Hemi TG, 5–6 weeks old) and homozygous (Homo TG, 5–6 weeks old) CD46TG mice, respectively. Bone marrow-derived dendritic cells and peritoneal macrophages were incubated with fluorescein isothiocyanate-conjugated anti-human CD46 antibody (E4.3; Pharmingen, San Diego, CA, USA) after incubation with anti-FcγRII/III monoclonal antibody (2.4G2; Pharmingen) to block nonspecific binding of the anti-human CD46 antibody. After being washed thoroughly, 10^4 stained cells were analyzed using a FACSCalibur (Becton Dickinson, Tokyo, Japan) and CellQuest software (Becton Dickinson).

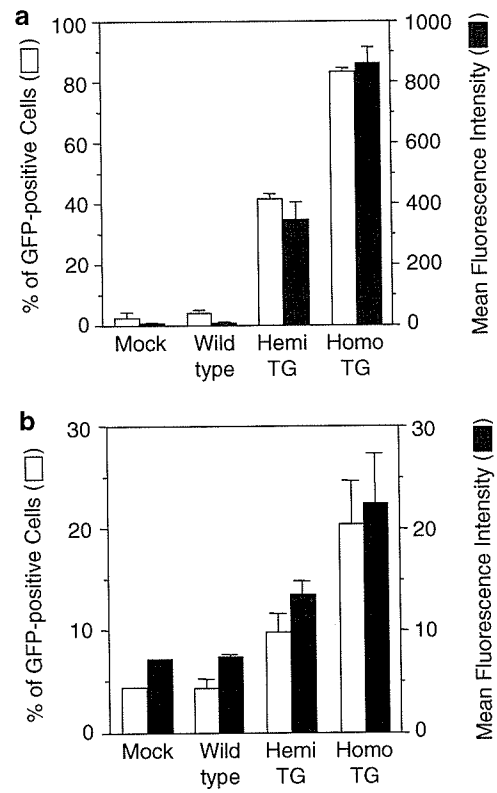


Figure 3 Ad35 vector-mediated green fluorescence protein (GFP) expression in (**a**) bone marrow-derived dendritic cells (mBM-DC) and (**b**) peritoneal macrophages prepared from wild-type mice and CD46TG mice. Open bars represent percentages of cells positive for GFP, and closed bars indicate mean fluorescence intensity. Bone marrow-derived dendritic cells (5×10^5 cells/well) and peritoneal macrophages (2×10^5 cells/well) prepared from wild-type mice and hemizygous (Hemi TG) and homozygous (Homo TG) CD46TG mice were seeded into 12-well plates the day before transduction. The cells were then transduced with Ad35GFP at 3000 VP/cell for 1.5 h. After a total of 48 h of incubation, GFP expression in cells was evaluated by flow cytometric analysis. The results are represented as mean ± s.d. ($n = 3$).

between the RGD motif in the penton base and αv-integrins, even in the absence of the primary receptor for Ad5, CAR.[36]

Next, to assess the *in vivo* transduction properties of Ad35 vectors in CD46TG mice, we intravenously and intraperitoneally administered an Ad35 vector that expressed luciferase (Ad35L). Ad35L was prepared in the same way as Ad35GFP. The PFU-to-particle ratio of Ad35L in 293-E1B cells was 1:625. Intravenous administration of Ad35L to hemizygous and homozygous CD46TG mice increased the transduction efficiencies for the liver, lung and kidney over those in wild-type mice. In contrast, no apparent increase in luciferase production occurred in the spleen or thymus of CD46TG mice (Figure 4a). There were no clear differences in the transduction efficiencies of Ad35 vectors between the homozygous and hemizygous mice, except in the case of liver.

In contrast, transduction efficiencies of Ad35L in the liver, spleen and kidney of CD46TG mice after intraperitoneal administration were much higher than those after intravenous administration (Figure 4b). Intraperitoneal injection into homozygous mice led to transduction efficiencies in the liver and kidney that were 83 and 271 times that after intravenous administration, respectively. Furthermore, even larger differences in transduction efficiency occurred between CD46TG mice and wild-type mice after intraperitoneal injection. Luciferase production from the liver, kidney and diaphragm of homozygous mice was 536, 492 and 83 times, respectively, that of wild-type mice. Comparison of the homozygous and hemizygous mice demonstrated that after intraperitoneal administration of vector, the homozygous mice appeared to be more susceptible to Ad35 vectors. Transgene expression levels in the liver and diaphragm of homozygous mice were 6.8 and 5.5 times, respectively, those of the hemizygous mice. The increased transduction efficiencies in the homozygous mice are probably owing to their increased levels of CD46 expression (Figure 1). These results indicate that CD46TG mice are more susceptible to Ad35 vectors than wild-type mice. However, the transduction efficiencies of Ad35 vectors in CD46TG mice after both intraperitoneal and intravenous injection were much lower than those of conventional Ad5 vectors in wild-type mice.[13] Luciferase production in the liver and spleen by Ad35 vectors intravenously administered to homozygous CD46TG mice was approximately 20 000 and 57 times lower, respectively, than that from Ad5 vectors administered intravenously to wild-type mice at the same dose as for the Ad35 vector in the present study (Ad5 vector-mediated luciferase expression levels in wild-type mice at a dose of 1.5×10^{10} VP/mouse after intravenous administration: liver, 2266 pg/mg protein; spleen, 0.893 pg/mg protein; kidney, 0.768 pg/mg protein; heart, 2.13 pg/mg protein; lung, 0.252 pg/mg protein).[13]

Next, to compare the fate of Ad35 vectors after *in vivo* administration to wild-type versus CD46TG mice, we used real-time PCR at 48 h post-administration to measure the amounts of Ad35L genomic sequences that had accumulated in various organs. After intravenous administration to CD46TG mice, the vector DNA in the sampled organs of CD46TG mice (except for liver) exceeded that in those of wild-type mice (Figure 5a). In addition, homozygous mice appeared to take up higher amounts of Ad35L than hemizygous mice. For example,

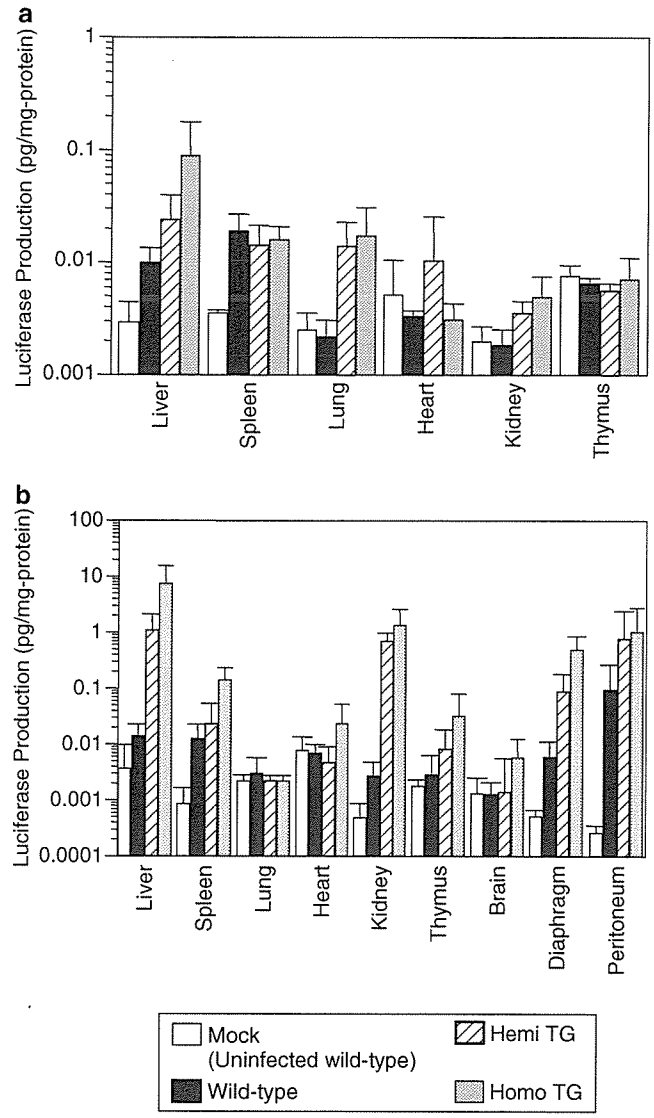


Figure 4 Luciferase production in CD46TG and wild-type mice after intravenous and intraperitoneal administration of Ad35L. (**a**) Luciferase production after intravenous administration of the vector. (**b**) Luciferase production after intraperitoneal administration. Ad35L (1.5×10^{10} VP) was administered to wild-type mice (C57Bl6, 5 weeks old) and hemizygous (Hemi TG, 5–6 weeks old) and homozygous (Homo TG, 5–6 weeks old) CD46TG mice. After 48 h, the organs were harvested and homogenized as described previously,[53] and luciferase production was measured by a luminescence assay system (PicaGene 5500; Toyo Inki, Japan). The data are represented as mean ± s.d. ($n = 4$, intravenous administration; $n = 6$, intraperitoneal administration).

Ad35 vector quantities in the spleen, which contained the most Ad35 vector DNA among the organs tested, of hemizygous and homozygous mice were 8 and 69 times, respectively, that in wild-type mice. In contrast, Ad35 vector DNA did not accumulate to high levels in the liver of CD46TG mice. Moreover, these quantities were similar, or slightly lower than, those in other organs of CD46TG mice, even though liver is well known to be a predominant organ for sequestration of Ad5 vectors administered intravenously to mice.[37,38]

After intraperitoneal injection, Ad35L was accumulated more efficiently in the liver, kidney, peritoneum

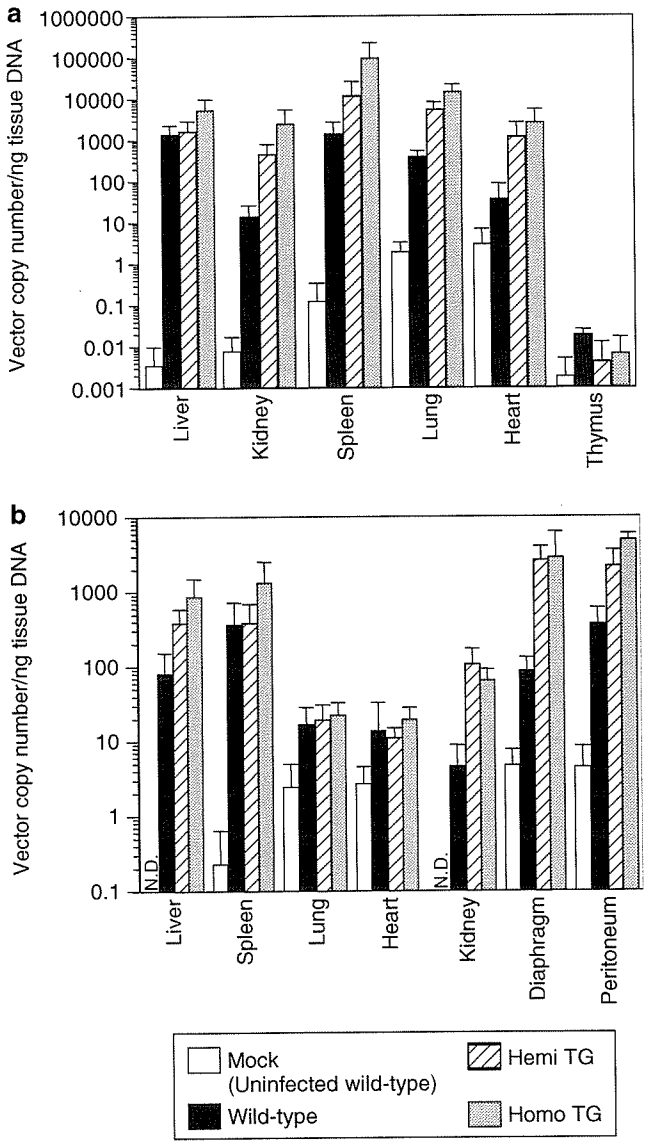


Figure 5 Tissue distribution of viral DNA in CD46TG and wild-type mice after intravenous and intraperitoneal administration of Ad35L. (**a**) Ad35L vector DNA detected in organs after intravenous administration. (**b**) Ad35L vector DNA detected in organs after intraperitoneal administration. Ad35L (1.5×10^{10} VP) was administered to wild-type mice (C57Bl6, 5 weeks old) and hemizygous (Hemi TG, 5–6 weeks old) and homozygous (Homo TG, 5–6 weeks old) CD46TG mice. After 48 h, the organs were harvested, and total DNA including viral DNA was extracted from the tissues after proteinase K digestion, and 25 ng samples of total DNA were subjected to quantitative real-time PCR, as described previously.[22] The data are represented as mean ± s.d. ($n = 4$).

and diaphragm of CD46TG mice than in those of wild-type mice (Figure 5b). The quantities of vector DNA in the liver and kidney of homozygous CD46TG mice were 11 and 14 times, respectively, those of wild-type mice. Furthermore, Ad35 vector DNA tended to be accumulated more efficiently in the liver, spleen, peritoneum and diaphragm of the homozygous mice than in those of the hemizygous mice. In addition, low levels of viral DNA were detected in the lung and heart, which are not directly accessible to intraperitoneally injected Ad35L from the injection point, with no significant difference between CD46TG mice and wild-type mice in the amounts that were detected. The data on the *in vivo* transduction efficiencies and viral DNA accumulation indicate that Ad35 vectors administered *in vivo* use human CD46 in CD46TG mice. In both intravenous and intraperitoneal dosing experiments, the total amounts of Ad35 vector DNA recovered from wild-type mice were lower than those from CD46TG mice. The decreased recovery of Ad35 vector DNA from wild-type mice could be due to its degradation in phagocytic cells, such as liver Kupffer cells. We speculate that the absence of human CD46 expression in organs results in decreased infection of the organs and increased uptake of Ad35 vector by phagocytic cells, leading to degradation of Ad35 vector DNA. In the previous study, we demonstrated that Ad35 vectors predominantly were taken up by liver nonparenchymal cells (endothelial and Kupffer cells) after intravenous administration in wild-type mice, and that the internalized Ad35 vector DNA was degraded rapidly.[13]

Finally, to examine the types of cells that Ad35 vectors transduce in CD46TG mice, we performed X-gal staining of the peritoneal organs after intraperitoneal administration of Ad35LacZ, an Ad35 vector expressing β-galactosidase (dose, 7.5×10^{10} VP/mouse). The vector was prepared by means of an improved *in vitro* ligation method[22,33,34] using pAdMS18 and pHMCMV6-LacZ. pAdMS18 was constructed by ligating oligonucleotides encoding I-*Ceu*I/*Swa*I/PI-*Sce*I into the *Pac*I site of pFS2-Ad35–7.[12] pHMCMV6-LacZ was generated by cloning the *Escherichia coli* β-galactosidase gene derived from pCMVβ (Clontech, Palo Alto, CA, USA) into the multicloning site of pHMCMV6.[34] The PFU-to-particle ratio of Ad35LacZ in 293-E1B cells was 1:315.

The peritoneal organs (liver, kidney and peritoneum) were efficiently transduced with Ad35LacZ (Figure 6a–f). However, X-gal staining of liver and kidney sections revealed that mainly the mesothelial cells on the surface of the liver and kidney were transduced; few deeper cells were transduced (Figure 6g–j). These results indicate that after injection, Ad35 vectors directly access the mesothelial cells of these organs, leading to efficient transduction.

In the present study, we assessed the *in vitro* and *in vivo* transduction properties of Ad35 vectors by using homozygous and hemizygous human CD46TG mice, a small animal model in which human CD46 is expressed with human-like tissue specificity. Human CD46 serves as a cellular receptor for not only subgroup B Ads but also several human pathogens, including measles virus, human herpes virus 6 and two types of bacteria.[39] Therefore, CD46TG mice already have been used in several studies, which have reported that replication of the pathogens and inflammatory responses occur in CD46TG mice after exposure to the pathogens, demonstrating the utility of CD46TG mice as animal models (note that for study of measles virus, the alpha/beta-interferon receptor gene is usually knocked out with insertion of the human CD46 gene).[28,40,41] In addition, in most of the CD46TG mice lines used in these studies, including in our present study, expression of human CD46 is driven by the human CD46 promoter, not the promoter of a ubiquitously expressed gene, leading to a pattern of CD46 expression similar to that in humans.[27,28,40] This conservation of the expression pattern is another advantage of using CD46TG mice as an animal model.

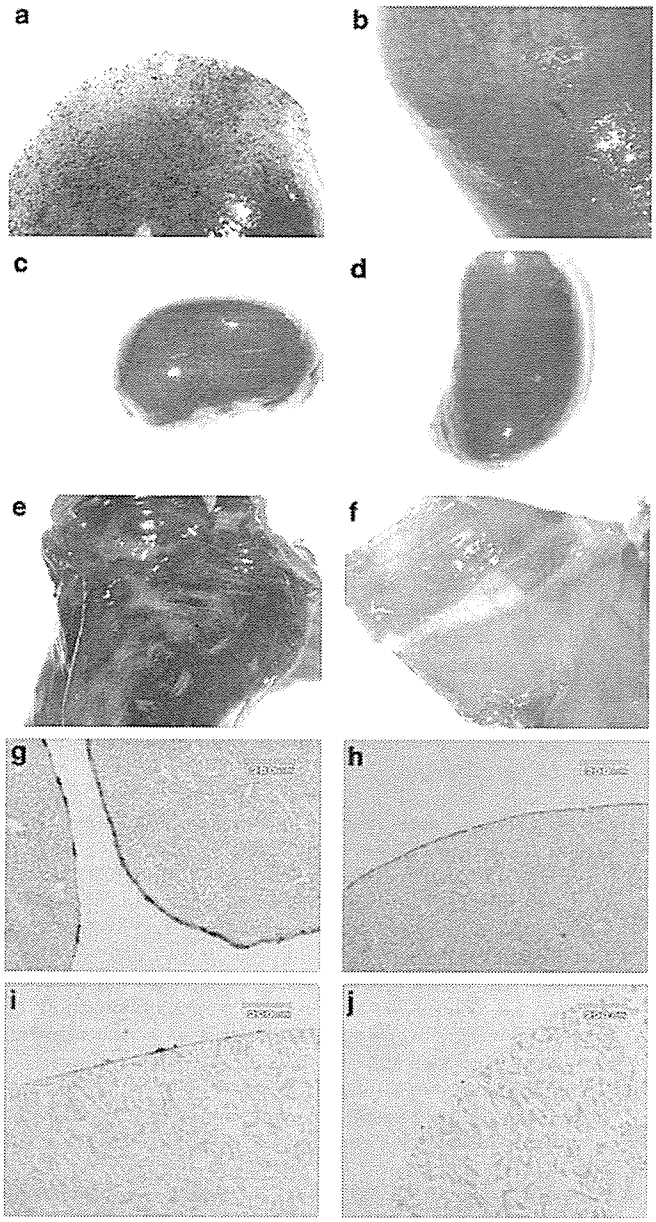


Figure 6 X-gal staining of the peritoneal organs of homozygous CD46TG mice receiving β-galactosidase-expressing Ad35 vectors. (**a**) Liver, (**c**) kidney, (**e**) peritoneum, (**g**) liver and (**i**) kidney sections from homo TG mice injected with Ad35LacZ. (**b**) Liver, (**d**) kidney, (**f**) peritoneum, (**h**) liver and (**j**) kidney sections from mock-infected homo TG mice. Ad35LacZ was injected intraperitoneally into homozygous CD46TG mice at a dose of 7.5×10^{10} VP/mouse. At 2 days postadministration, the organs were recovered after perfusion with 0.5% glutaraldehyde solution and then fixed and stained as described previously[54] by using 0.5% glutaraldehyde instead of 4% paraformaldehyde. For X-gal staining of liver and kidney, 10 μm sections were cut, fixed with 0.5% glutaraldehyde, and stained as described previously.[55]

In vivo transduction experiments using CD46TG mice showed that Ad35 vectors mediated higher transduction efficiencies in CD46TG mice than wild-type mice, indicating that Ad35 vectors recognize human CD46 as an attachment receptor on *in vivo* application. However, the transduction efficiencies of Ad35 vectors in CD46TG mice were much lower than expected. Addition of human CD46 expression to mouse primary cells greatly enhanced Ad35 vector-mediated transduction (Figure 3). Our previous study demonstrated that the transduction activities of Ad5 and Ad35 vectors were nearly equivalent in human cultured cell lines,[13] which express high levels of CD46. These results suggest that, in cells expressing sufficient CD46, the transduction efficiencies of Ad35 vectors could be similar to those of Ad5 vectors. Therefore, we had expected that the transduction efficiencies of Ad35 vectors in CD46TG mice would greatly increase to levels comparable to those of Ad5 vectors. However, the transduction efficiencies of Ad35 vectors in the organs of homozygous CD46TG mice after intravenous administration were approximately 20- to 20 000-fold lower than those of conventional Ad5 vectors at the same dose (1.5×10^{10} VP/mouse) in wild-type mice.[13] Why the *in vivo* transduction efficiencies of Ad35 vectors in CD46TG mice were lower than expected remains to be clarified. One possibility is that Ad35 vectors administered to CD46TG mice cannot access the human CD46 that is expressed on the cells. Human CD46 primarily is expressed on the basolateral surfaces of polarized epithelial cells,[42,43] and measles virus preferentially infects cells from their basolateral, rather than apical, sides.[44] Anatomical barriers such as the tightness between the basal membrane and extracellular matrix might impair the access of Ad35 vectors to the human CD46 on the basolateral cell surface. Another possibility is that an unidentified receptor or co-receptor for Ad35 is expressed in humans but not in CD46TG mice. Segerman *et al.*[45] suggest that there are two different receptors in human cells for subgroup B Ads. Interaction between Ad35 vectors and blood components (blood cells or serum proteins) might also inhibit Ad35 vector-mediated transduction after intravenous administration. In particular, the soluble form of human CD46, which is found in normal human serum,[46] might block infection by Ad35 vectors. However, preincubation of Ad35 vectors with serum or blood cells recovered from CD46TG mice did not reduce the transduction efficiencies of Ad35 vectors *in vitro* (data not shown). Further evaluation is necessary to clarify whether studies using CD46TG mice appropriately evaluate the transduction properties of Ad35 vectors. Currently, we are examining the transduction properties of Ad35 vectors in non-human primates, which express CD46 in all organs. The findings should greatly help us to understand characteristics of Ad35 vector-mediated transduction, including the validity and utility of CD46TG mice as model animals for Ad35 vectors.

The fiber shaft of Ad35 lacks the KKTK (Lys-Lys-Thr-Lys) motif, which is located in the fiber shaft of Ad5 and is considered to bind to heparan sulfate,[47] and its absence may partly explain the lower transduction efficiencies of Ad35 vectors than Ad5 vectors. Smith *et al.*[47] demonstrated that amino-acid substitution of the KKTK motif dramatically decreased the transduction efficiencies of Ad5 vectors in the mouse liver, whereas ablation of CAR- and integrin-binding sites did not significantly reduce the transduction efficiency of Ad5 vectors in the liver; these findings indicate a potential role for heparan sulfate binding in Ad5 vector-mediated liver transduction. In addition, we reported that replacement of the Ad5 fiber shaft with the Ad35 shaft, in addition to the ablation of CAR and integrin binding, decreases Ad5 transduction efficiencies in the liver.[48] Heparan sulfate

binding may be a more crucial determinant for Ad vector-mediated *in vivo* transduction of mice, especially in the liver, than binding to primary receptors (CAR for Ad5 and CD46 for Ad35), irrespective of Ad5 or Ad35 vectors.

Homozygous CD46TG mice, which express CD46 more abundantly than hemizygous CD46TG mice, seem to be more susceptible to Ad35 vectors than hemizygous mice (Figure 4). Our *in vitro* transduction experiments that used primary cells from CD46TG mice also demonstrated that mBM-DC and peritoneal macrophages derived from wild-type mice were refractory to Ad35 vectors, but high-level CD46 expression in cells from CD46TG mice increased the transduction efficiencies of Ad35 vectors (Figure 3). Together, these results indicate that CD46 expression levels are a crucial factor in Ad35 vector-mediated transduction. Anderson *et al.*[49] reported a similar result, in which the transduction efficiencies of the chimeric Ad5F35 vector, which is an Ad5-based vector containing an Ad35 fiber shaft and knob, increased progressively with CD46 expression density in a panel of CHO cells stably expressing CD46.[49] Together, these findings suggest that Ad35 vectors can be a potent and selective platform for transduction into tumor cells expressing high levels of CD46, because human malignant tumor cells (including primary tumor cells) express CD46 more abundantly than nontransformed human cells.[23,24] Engineered measles viruses, which enter cells efficiently via CD46, exhibit selective oncolytic activity by exploiting the difference in CD46 expression levels between ovarian cancer cells and nontransformed cells.[23]

Transduction efficiencies of intraperitoneal-administered Ad35 vectors were >10-fold higher in homozygous CD46TG mice than in wild-type mice (Figure 4). In particular, peritoneal organs, such as the liver, kidney, peritoneal wall and diaphragm, were transduced efficiently. X-gal staining experiments demonstrated that efficient transduction occurred mainly in the mesothelial cells of the liver and kidney (Figure 6). In contrast, noteworthy luciferase production after intraperitoneal administration of Ad35 vector was not detected in the lung or heart, which are distant from the peritoneal cavity. Few LacZ-positive cells were found in the interior of the liver and kidney. These results indicate that after intraperitoneal injection, a portion of the Ad35 vector dose efficiently infects the mesothelia of tissues via CD46 on the surface of the organ, leading to efficient transduction. Another fraction of the dose, which is probably exuded into the bloodstream from the peritoneal cavity, likely poorly transduces the tissues of CD46TG mice. Using quantitative real-time PCR, we confirmed that Ad35 vector DNA was present in the blood after intraperitoneal injection (data not shown).

In summary, we demonstrated that Ad35 vectors used the human CD46 expressed in CD46TG mice as an attachment receptor after *in vivo* administration. CD46TG mice are more susceptible to Ad35 vector-mediated transduction than wild-type mice, but the transduction efficiencies of Ad35 vectors in CD46TG mice were much lower than those of conventional Ad5 vectors.[13] As a next step, evaluation of Ad35 vector-mediated transduction in non-human primates, which ubiquitously express CD46 (as do humans), is necessary to assess the validity and utility of CD46TG mice as small animal models for Ad35 vectors.

Acknowledgements

We thank Ms Tomomi Sasaki, Ms Tsukasa Nakano and Ms Kimiyo Akitomo for their technical assistance. We also thank Dr Naoki Okada (Kyoto Pharmaceutical University, Kyoto, Japan) for his help in the preparation of bone marrow-derived DCs. This work was supported by grants for Health and Labour Sciences Research from the Ministry of Health, Labour, and Welfare of Japan, and by Grants-in-Aid for Scientific Research on Priority Areas (B).

References

1 Havenga MJ, Lemckert AA, Ophorst OJ, van Meijer M, Germeraad WT, Grimbergen J *et al.* Exploiting the natural diversity in adenovirus tropism for therapy and prevention of disease. *J Virol* 2002; **76**: 4612–4620.
2 De Jong JC, Wermenbol AG, Verweij-Uijterwaal MW, Slaterus KW, Wertheim-Van Dillen P, Van Doornum GJ *et al.* Adenoviruses from human immunodeficiency virus-infected individuals, including two strains that represent new candidate serotypes Ad50 and Ad51 of species B1 and D, respectively. *J Clin Microbiol* 1999; **37**: 3940–3945.
3 Chirmule N, Propert K, Magosin S, Qian Y, Qian R, Wilson J. Immune responses to adenovirus and adeno-associated virus in humans. *Gene Therapy* 1999; **6**: 1574–1583.
4 Stone D, Ni S, Li ZY, Gaggar A, DiPaolo N, Feng Q *et al.* Development and assessment of human adenovirus type 11 as a gene transfer vector. *J Virol* 2005; **79**: 5090–5104.
5 Smith TA, White BD, Gardner JM, Kaleko M, McClelland A. Transient immunosuppression permits successful repetitive intravenous administration of an adenovirus vector. *Gene Therapy* 1996; **3**: 496–502.
6 Vlachaki MT, Hernandez-Garcia A, Ittmann M, Chhikara M, Aguilar LK, Zhu X *et al.* Impact of preimmunization on adenoviral vector expression and toxicity in a subcutaneous mouse cancer model. *Mol Ther* 2002; **6**: 342–348.
7 Rebel VI, Hartnett S, Denham J, Chan M, Finberg R, Sieff CA. Maturation and lineage-specific expression of the coxsackie and adenovirus receptor in hematopoietic cells. *Stem Cells* 2000; **18**: 176–182.
8 Tillman BW, de Gruijl TD, Luykx-de Bakker SA, Scheper RJ, Pinedo HM, Curiel TJ *et al.* Maturation of dendritic cells accompanies high-efficiency gene transfer by a CD40-targeted adenoviral vector. *J Immunol* 1999; **162**: 6378–6383.
9 Haviv YS, Blackwell JL, Kanerva A, Nagi P, Krasnykh V, Dmitriev I *et al.* Adenoviral gene therapy for renal cancer requires retargeting to alternative cellular receptors. *Cancer Res* 2002; **62**: 4273–4281.
10 Abrahamsen K, Kong HL, Mastrangeli A, Brough D, Lizonova A, Crystal RG *et al.* Construction of an adenovirus type 7a E1A- vector. *J Virol* 1997; **71**: 8946–8951.
11 Holterman L, Vogels R, van der Vlugt R, Sieuwerts M, Grimbergen J, Kaspers J *et al.* Novel replication-incompetent vector derived from adenovirus type 11 (Ad11) for vaccination and gene therapy: low seroprevalence and non-cross-reactivity with Ad5. *J Virol* 2004; **78**: 13207–13215.
12 Sakurai F, Mizuguchi H, Hayakawa T. Efficient gene transfer into human CD34+ cells by an adenovirus type 35 vector. *Gene Therapy* 2003; **10**: 1041–1048.
13 Sakurai F, Mizuguchi H, Yamaguchi T, Hayakawa T. Characterization of *in vitro* and *in vivo* gene transfer properties of adenovirus serotype 35 vector. *Mol Ther* 2003; **8**: 813–821.
14 Vogels R, Zuijdgeest D, van Rijnsoever R, Hartkoorn E, Damen I, de Bethune MP *et al.* Replication-deficient human adenovirus

type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity. *J Virol* 2003; **77**: 8263–8271.
15 Seshidhar Reddy P, Ganesh S, Limbach MP, Brann T, Pinkstaff A, Kaloss M *et al*. Development of adenovirus serotype 35 as a gene transfer vector. *Virology* 2003; **311**: 384–393.
16 Farina SF, Gao GP, Xiang ZQ, Rux JJ, Burnett RM, Alvira MR *et al*. Replication-defective vector based on a chimpanzee adenovirus. *J Virol* 2001; **75**: 11603–11613.
17 Reddy PS, Idamakanti N, Chen Y, Whale T, Babiuk LA, Mehtali M *et al*. Replication-defective bovine adenovirus type 3 as an expression vector. *J Virol* 1999; **73**: 9137–9144.
18 Nguyen T, Nery J, Joseph S, Rocha C, Carney G, Spindler K *et al*. Mouse adenovirus (MAV-1) expression in primary human endothelial cells and generation of a full-length infectious plasmid. *Gene Therapy* 1999; **6**: 1291–1297.
19 Hofmann C, Loser P, Cichon G, Arnold W, Both GW, Strauss M. Ovine adenovirus vectors overcome preexisting humoral immunity against human adenoviruses *in vivo*. *J Virol* 1999; **73**: 6930–6936.
20 Gaggar A, Shayakhmetov DM, Lieber A. CD46 is a cellular receptor for group B adenoviruses. *Nat Med* 2003; **9**: 1408–1412.
21 Segerman A, Atkinson JP, Marttila M, Dennerquist V, Wadell G, Arnberg N. Adenovirus type 11 uses CD46 as a cellular receptor. *J Virol* 2003; **77**: 9183–9191.
22 Sakurai F, Kawabata K, Yamaguchi T, Hayakawa T, Mizuguchi H. Optimization of adenovirus serotype 35 vectors for efficient transduction in human hematopoietic progenitors: comparison of promoter activities. *Gene Therapy* 2005; **12**: 1424–1433.
23 Peng KW, TenEyck CJ, Galanis E, Kalli KR, Hartmann LC, Russell SJ. Intraperitoneal therapy of ovarian cancer using an engineered measles virus. *Cancer Res* 2002; **62**: 4656–4662.
24 Bjorge L, Hakulinen J, Wahlstrom T, Matre R, Meri S. Complement-regulatory proteins in ovarian malignancies. *Int J Cancer* 1997; **70**: 14–25.
25 Tsujimura A, Shida K, Kitamura M, Nomura M, Takeda J, Tanaka H *et al*. Molecular cloning of a murine homologue of membrane cofactor protein (CD46): preferential expression in testicular germ cells. *Biochem J* 1998; **330** (Part 1): 163–168.
26 Johnstone RW, Loveland BE, McKenzie IF. Identification and quantification of complement regulator CD46 on normal human tissues. *Immunology* 1993; **79**: 341–347.
27 Kemper C, Leung M, Stephensen CB, Pinkert CA, Liszewski MK, Cattaneo R *et al*. Membrane cofactor protein (MCP; CD46) expression in transgenic mice. *Clin Exp Immunol* 2001; **124**: 180–189.
28 Oldstone MB, Lewicki H, Thomas D, Tishon A, Dales S, Patterson J *et al*. Measles virus infection in a transgenic model: virus-induced immunosuppression and central nervous system disease. *Cell* 1999; **98**: 629–640.
29 Lemckert AA, Sumida SM, Holterman L, Vogels R, Truitt DM, Lynch DM *et al*. Immunogenicity of heterologous prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-ad5 immunity. *J Virol* 2005; **79**: 9694–9701.
30 Barouch DH, Pau MG, Custers JH, Koudstaal W, Kostense S, Havenga MJ *et al*. Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity. *J Immunol* 2004; **172**: 6290–6297.
31 Okada N, Masunaga Y, Okada Y, Iiyama S, Mori N, Tsuda T *et al*. Gene transduction efficiency and maturation status in mouse bone marrow-derived dendritic cells infected with conventional or RGD fiber-mutant adenovirus vectors. *Cancer Gene Ther* 2003; **10**: 421–431.
32 Takagi T, Kitano M, Masuda S, Tokuda H, Takakura Y, Hashida M. Augmented inhibitory effect of superoxide dismutase on superoxide anion release from macrophages by direct cationization. *Biochim Biophys Acta* 1997; **1335**: 91–98.
33 Mizuguchi H, Kay MA. Efficient construction of a recombinant adenovirus vector by an improved *in vitro* ligation method. *Hum Gene Ther* 1998; **9**: 2577–2583.
34 Mizuguchi H, Kay MA. A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. *Hum Gene Ther* 1999; **10**: 2013–2017.
35 Zubieta C, Schoehn G, Chroboczek J, Cusack S. The structure of the human adenovirus 2 penton. *Mol Cell* 2005; **17**: 121–135.
36 Mizuguchi H, Koizumi N, Hosono T, Ishii-Watabe A, Uchida E, Utoguchi N *et al*. CAR- or alphav integrin-binding ablated adenovirus vectors, but not fiber-modified vectors containing RGD peptide, do not change the systemic gene transfer properties in mice. *Gene Therapy* 2002; **9**: 769–776.
37 Wood M, Perrotte P, Onishi E, Harper ME, Dinney C, Pagliaro L *et al*. Biodistribution of an adenoviral vector carrying the luciferase reporter gene following intravesical or intravenous administration to a mouse. *Cancer Gene Ther* 1999; **6**: 367–372.
38 Huard J, Lochmuller H, Acsadi G, Jani A, Massie B, Karpati G. The route of administration is a major determinant of the transduction efficiency of rat tissues by adenoviral recombinants. *Gene Therapy* 1995; **2**: 107–115.
39 Cattaneo R. Four viruses, two bacteria, and one receptor: membrane cofactor protein (CD46) as pathogens' magnet. *J Virol* 2004; **78**: 4385–4388.
40 Mrkic B, Pavlovic J, Rulicke T, Volpe P, Buchholz CJ, Hourcade D *et al*. Measles virus spread and pathogenesis in genetically modified mice. *J Virol* 1998; **72**: 7420–7427.
41 Johansson L, Rytkonen A, Bergman P, Albiger B, Kallstrom H, Hokfelt T *et al*. CD46 in meningococcal disease. *Science* 2003; **301**: 373–375.
42 Maisner A, Zimmer G, Liszewski MK, Lublin DM, Atkinson JP, Herrler G. Membrane cofactor protein (CD46) is a basolateral protein that is not endocytosed. Importance of the tetrapeptide FTSL at the carboxyl terminus. *J Biol Chem* 1997; **272**: 20793–20799.
43 Ichida S, Yuzawa Y, Okada H, Yoshioka K, Matsuo S. Localization of the complement regulatory proteins in the normal human kidney. *Kidney Int* 1994; **46**: 89–96.
44 Sinn PL, Williams G, Vongpunsawad S, Cattaneo R, McCray Jr PB. Measles virus preferentially transduces the basolateral surface of well-differentiated human airway epithelia. *J Virol* 2002; **76**: 2403–2409.
45 Segerman A, Arnberg N, Erikson A, Lindman K, Wadell G. There are two different species B adenovirus receptors: sBAR, common to species B1 and B2 adenoviruses, and sB2AR, exclusively used by species B2 adenoviruses. *J Virol* 2003; **77**: 1157–1162.
46 Seya T, Hara T, Iwata K, Kuriyama S, Hasegawa T, Nagase Y *et al*. Purification and functional properties of soluble forms of membrane cofactor protein (CD46) of complement: identification of forms increased in cancer patients' sera. *Int Immunol* 1995; **7**: 727–736.
47 Smith TA, Idamakanti N, Rollence ML, Marshall-Neff J, Kim J, Mulgrew K *et al*. Adenovirus serotype 5 fiber shaft influences *in vivo* gene transfer in mice. *Hum Gene Ther* 2003; **14**: 777–787.
48 Koizumi N, Mizuguchi H, Sakurai F, Yamaguchi T, Watanabe Y, Hayakawa T. Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and alphav integrin-binding ablation. *J Virol* 2003; **77**: 13062–13072.
49 Anderson BD, Nakamura T, Russell SJ, Peng KW. High CD46 receptor density determines preferential killing of tumor cells by oncolytic measles virus. *Cancer Res* 2004; **64**: 4919–4926.
50 Kimura Y, Yanagimachi R. Intracytoplasmic sperm injection in the mouse. *Biol Reprod* 1995; **52**: 709–720.

51 Perry AC, Wakayama T, Kishikawa H, Kasai T, Okabe M, Toyoda Y *et al.* Mammalian transgenesis by intracytoplasmic sperm injection. *Science* 1999; **284**: 1180–1183.
52 Hosono T, Mizuguchi H, Katayama K, Xu ZL, Sakurai F, Ishii-Watabe A *et al.* Adenovirus vector-mediated doxycycline-inducible RNA interference. *Hum Gene Ther* 2004; **15**: 813–819.
53 Xu ZL, Mizuguchi H, Ishii-Watabe A, Uchida E, Mayumi T, Hayakawa T. Optimization of transcriptional regulatory elements for constructing plasmid vectors. *Gene* 2001; **272**: 149–156.
54 Sakai M, Nishikawa M, Thanaketpaisarn O, Yamashita F, Hashida M. Hepatocyte-targeted gene transfer by combination of vascularly delivered plasmid DNA and *in vivo* electroporation. *Gene Therapy* 2005; **12**: 607–616.
55 Sakurai F, Nishioka T, Saito H, Baba T, Okuda A, Matsumoto O *et al.* Interaction between DNA–cationic liposome complexes and erythrocytes is an important factor in systemic gene transfer via the intravenous route in mice: the role of the neutral helper lipid. *Gene Therapy* 2001; **8**: 677–686.

ARTICLE

doi:10.1016/j.ymthe.2005.10.007

Quantitative Comparison of Intracellular Trafficking and Nuclear Transcription between Adenoviral and Lipoplex Systems

Susumu Hama,[1,2] Hidetaka Akita,[1,2] Rie Ito,[1] Hiroyuki Mizuguchi,[3] Takao Hayakawa,[4] and Hideyoshi Harashima[1,2,*]

[1]*Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Hokkaido 060-0812, Japan*
[2]*CREST, Japan Science and Technology Corporation, Tokyo, Japan*
[3]*Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka 567-0085, Japan*
[4]*Pharmaceuticals and Medical Devices Agency, Tokyo 100-0013, Japan*

**To whom correspondence and reprint requests should be addressed. Fax: +81 11 706 4879. E-mail: harasima@pharm.hokudai.ac.jp.*

Available online 20 December 2005

To develop nonviral gene vectors that are sufficient for clinical application, it is necessary to understand why and to what extent nonviral vectors are inferior to viral vectors, which in general show a more efficient transfection activity. This study describes a systematic and quantitative comparison of the cellular uptake and subsequent intracellular distribution (e.g., endosome/lysosome, cytosol, and nucleus) of exogenous DNA transfected by viral and nonviral vectors in living cells, using a combination of TaqMan PCR and a recently developed confocal image-assisted three-dimensionally integrated quantification method. As a model, adenovirus (Ad) and Lipofectamine Plus (LFN) were used for comparison since they are highly potent and widely used viral and nonviral vectors, respectively. The findings indicate that the efficiency of cellular uptake for LFN is significantly higher than that for Ad. Once taken up by a cell, Ad exhibited comparable endosomal escape and slightly higher nuclear transfer efficiency compared with LFN. In contrast, LFN requires 3 orders of magnitude more intranuclear gene copies to exhibit a transgene expression comparable to that of the Ad, suggesting that the difference in transfection efficiency principally arises from differences in nuclear transcription efficiency and not from a difference in intracellular trafficking between Ad and LFN.

Key Words: adenovirus, nonviral vector, lipoplex, quantification, intracellular trafficking, gene vector

INTRODUCTION

Numerous nonviral vectors have been developed for use in gene therapy for intractable diseases [1,2]. However, low transfection efficiency still remains a bottleneck, preventing its use in clinical applications. It is generally considered that transfection activity is rate limited to a great extent, by a variety of intracellular processes such as endosomal escape, nuclear transfer, and intranuclear transcription.

Along with evolution of life during the past hundreds of millions of years, DNA and RNA viruses have also evolved and have developed sophisticated mechanisms for controlling intracellular trafficking for the efficient delivery of their genomes to nuclei in host cells for symbiosis. Although some nonviral vectors have been evolutionally developed since the first proposal of the concept of gene therapy over 30 years ago [3], this history is overwhelmingly short. Therefore, the transfection efficiency of a virus vector is, in general, more prominent than that of a nonviral vector [4]. To improve nonviral vectors, quantitative information concerning why and to what extent the nonviral vector is inferior to the viral one is essential.

For the quantification of intracellular trafficking, we and other researchers have developed methodology to quantify the amount of plasmid DNA in the nucleus by nuclear fractionation followed by the polymerase chain reaction (PCR) [5–7]. This quantification revealed an important lesson showing that it is necessary to optimize not only the nuclear delivery of plasmid DNA, but also intranuclear transcription efficiency, since transgene expression is remarkably saturated against nucleus-delivered pDNA. In contrast to the nucleus, very few reports are available concerning the amount of pDNA in the

1525-0016/$30.00

endosome/lysosome compartment, and therefore, it is very difficult to evaluate the efficiency of endosomal release. Although the subcellular fractionation of endosomes/lysosomes may solve this issue, many problems, such as the complicated protocol, uncertainties associated with the recovery of the endosomal fraction, and mutual contamination may prevent this strategy from becoming a practical application.

We recently developed a novel strategy, the confocal image-assisted three-dimensionally integrated quantification (CIDIQ) method, which enables the distribution of exogenous DNA in endosome/lysosome, cytosol, and nucleus to be quantified simultaneously in individual cells with sequential *Z*-series images captured by confocal laser scanning microscopy [8,9]. Since intracellular trafficking investigated by CIDIQ can readily explain the differences in transgene expression by various nonviral vectors, this method is useful for identifying the rate-limiting barriers to gene expression.

In the present study, we applied it to the systematic and quantitative comparison of the intracellular distribution of exogenous genes transfected by viral vector and nonviral vector in living cells. It enabled us to examine the rate-limiting processes associated with nonviral vectors that limit their transfection efficiency. As model vectors, we used adenovirus (Ad) and Lipofectamine Plus (LFN), highly potent and widely used viral and nonviral vectors (lipoplex), respectively, in the comparative study.

RESULTS

Comparison of Transfection Activity between Ad and LFN

We initially compared the time-dependent and applied dose-dependent transfection activity between Ad and LFN. The methodology used to determine the applied dose in terms of luciferase gene copies is described under Materials and Methods. As shown in Fig. 1A, transgene expression is increased in a dose-dependent manner for both vectors 6 h after incubation. In subsequent experiments, we fixed the dose of plasmid DNA (pDNA) in the LFN-mediated transfection at 6.7×10^5 copies/cell (5 pg/cell) based on the manufacturer's recommended protocol, from which approximately 5×10^7 RLU/mg protein of luciferase activity was exhibited. In the case of Ad, we fixed the dose at 200 copies/cell, since comparable levels of transgene expression can be achieved at this dose. As shown in Fig. 1B, both vectors exhibited quite comparable time courses for transgene expression at these doses, suggesting that LFN is a potent system for the delivery of pDNA to the nucleus with a speed comparable to that of Ad. However, it should be emphasized that LFN requires 3 orders of magnitude more gene copies than the Ad to achieve comparable gene expression (Fig. 1A). Furthermore, the difference in the required dose for achieving a comparable level of transgene expression is dependent on the expression level. In a comparison of the expression of 1×10^6 RLU/mg protein, approximately 10,000-fold more copies of plasmid DNA were required for LFN, and at a lower transgene expression level, the difference was even greater. This is due to the nonlinear relationship between the dose and the transfection efficiency in LFN and is also sometimes observed in various nonviral vectors [10].

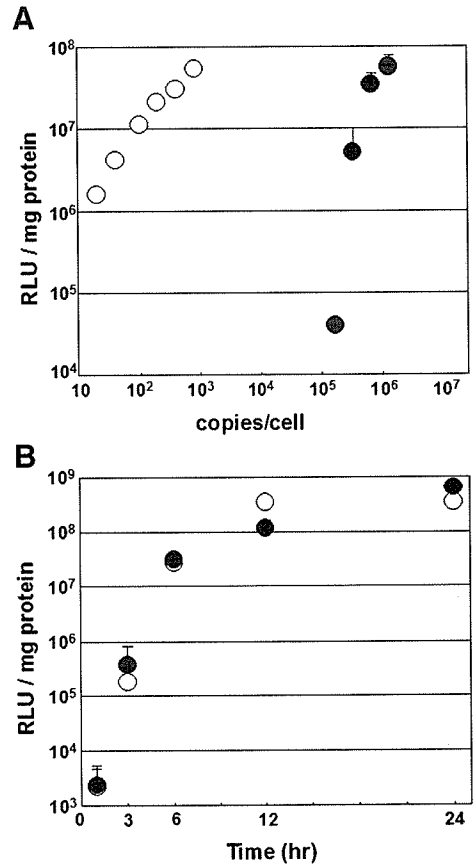


FIG. 1. Dose–response curve and time course of luciferase gene expression in A549 cells transfected by Ad and LFN. (A) Luciferase gene expression in cells transfected by Ad (○) or LFN (●) was measured 6 h after incubation at the indicated dose. (B) Transfection activities were measured at indicated times after incubation with a dose of 200 (○) or 6.7×10^5 copies/cell (5 pg/cell) (●). The vertical axis represents luciferase activity expressed as relative light units (RLU)/mg protein. These data represent the mean values and standard deviation of three experiments.

Quantification of an Intracellular Distribution of pDNA and Ad DNA

We then quantified the intracellular distribution of pDNA or Ad by a combination of TaqMan PCR and CIDIQ [8]. The procedures used to determine the distribution of exogenous DNA transfected by LFN and Ad are