Targeted Delivery of CX3CL1 to Multiple Lung Tumors by Mesenchymal Stem Cells

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ORIGINAL ARTICLE

Downregulation of human CD46 by adenovirus serotype 35 vectors

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Human CD46 (membrane cofactor protein), which serves as a receptor for a variety of pathogens, including strains of measles virus, human herpesvirus type 6 and Neisseria, *is rapidly downregulated from the cell surface following infection by these pathogens. Here, we report that replication-incompetent adenovirus (Ad) serotype 35 (Ad35) vectors, which belong to subgroup B and recognize human CD46 as a receptor, downregulate CD46 following infection. A decline in the surface expression of CD46 in human peripheral blood mononuclear cells was detectable 6 h after infection, and reached maximum (72%) 12 h after infection. Ad35 vector-induced downregulation of surface CD46 levels gradually recovered after the removal of Ad35 vectors, however, complete recovery of CD46 expression was not observed even at 96 h after removal. The surface expression of CD46 was also reduced after incubation with fiber-substituted Ad serotype 5 (Ad5) vectors bearing Ad35 fiber proteins, ultraviolet-irradiated Ad35, vectors and recombinant Ad35 fiber knob proteins; in contrast, conventional Ad5 vectors did not induce surface CD46 downregulation, suggesting that the fiber knob protein of Ad35 plays a crucial role in the downregulation of surface CD46 density. These results have important implications for gene therapy using CD46-utilizing Ad vectors and for the pathogenesis of Ads that interact with CD46.*
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Introduction

Human CD46 is a transmembrane glycoprotein, which is ubiquitously expressed in most or all human nucleated cells. CD46 functions as a regulator of complement activation, whose normal function is to protect the host from autologous complement attack, by binding complement components C3b and C4b and facilitating their cleavage by factor I.[1,2] In addition to these functions, CD46 serves as a receptor for several pathogens, including strains of measles virus (MV),[3] human herpesvirus type 6 (HHV6),[4] group A *streptococci*[5] and *Neisseria*.[6] Among these pathogens, infection by certain strains of MV,[7,8] HHV6[4] and *Neisseria gonorrhoeae*[9] has been shown to cause CD46 downregulation from the cell surface. The detailed mechanisms of surface CD46 downregulation upon infection by these pathogens remain to be elucidated, however, the decrease in the surface density of CD46 renders the cells more susceptible to lysis by complements, as demonstrated *in vitro*,[10] and may contribute to the attenuation of these pathogens by rapid clearing of infected cells.

Recently, it has been demonstrated that CD46 also acts as a receptor for the majority of subgroup B adenoviruses (Ads), including Ad serotypes 11 (Ad11) and 35 (Ad35).[11,12] The fiber knob domain of Ad11 or Ad35 binds to short consensus repeats (SCRs) 1 and/or 2 in CD46 for infection.[13–15] Furthermore, Ad35 competes for binding to CD46 with the MV hemagglutinin (MVH) protein,[14] which is responsible for both the attachment of MV to CD46[16] and downregulation of surface CD46 expression levels.[17] These findings led us to hypothesize that CD46 is downregulated following infection by subgroup B Ads, as occurs in the case of MV. On the other hand, subgroup B Ad11 and Ad35 are considered to be an attractive framework for gene transfer vectors for the following reasons. First, Ad11 and Ad35 are known to be rarely neutralized by human sera.[18] Second, Ad11 and Ad35 exhibit a broad tropism including cells expressing no or low levels of coxsackievirus and adenovirus receptor (CAR), which is a receptor for Ads belonging to subgroups A, C, D, E and F.[19] Several groups (including the authors) have developed replication-incompetent Ad vectors composed of subgroup B Ads[20–24] or fiber-substituted Ad serotype 5 (Ad5) vectors containing subgroup B Ad fibers,[25–28] and have demonstrated that these types of Ad vectors efficiently transduce a variety of human cells, including cells refractory to conventional Ad5 vectors. If surface CD46 downregulation occurs following transduction with CD46-utilizing Ad vectors, unexpected side effects might occur such as complement-mediated cell lysis of successfully transduced cells, which leads to clearance of the transduced cells.

In the present study, we examined replication-incompetent Ad35 vector-induced downregulation of surface

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CD46 expression. We found that transduction with Ad35 vectors significantly downregulated CD46 expression from the cell surface in a dose-dependent and cell type-specific manner. Ad35 vector-mediated downregulation of surface CD46 was found to occur in leukemia cells, whereas nonleukemia cells did not exhibit any decline in surface CD46 expression following Ad35 vector infection. To the best of our knowledge, this is the first report characterizing subgroup B Ad-mediated downregulation of surface CD46.

Results

Infection with Ad35 vectors causes downregulation of surface CD46 expression

To determine whether infection with Ad35 vectors results in modulation of surface CD46 expression, human peripheral blood mononuclear cells (PBMCs) were incubated with the Ad35 vector expressing green fluorescence protein (GFP) (Ad35GFP) at 10 000 vector particle (VP)/cell and subjected to flow cytometric analysis at various time points. This analysis demonstrated that the surface expression levels of CD46 in PBMCs gradually decreased during exposure to Ad35GFP (Figure 1a). The significant decrease in CD46 was detectable 6 h after infection and reached maximum 12 h after infection (72% downregulation). Furthermore, the downregulation of surface CD46 by Ad35 vectors was found to be dose dependent (Figure 1b). PBMCs infected at 1250 VP/cell showed significantly reduced levels of CD46 expression (44% downregulation), and 71% downregulation of surface CD46 expression was induced at 20 000 VP/cell. These results indicate that infection with Ad35 vectors downregulates surface CD46 expression, as happens in the cases of MV[7,8] and HHV6.[4] The viability of PBMCs was not significantly affected by Ad35 vector infection (data not shown).

Next, in order to examine whether B cells ($CD19^+$ cells) and T cells ($CD3^+$ cells) in PBMCs show a reduction in surface CD46 levels after infection with Ad35 vectors, PBMCs were simultaneously stained with anti-human CD46 and anti-human CD19 or anti-human CD3 antibodies, and were subsequently subjected to flow cytometric analysis. Surface CD46 downregulation was found in both B cells and T cells after Ad35 vector infection, but the levels of the downregulation in these cells were lower than those of whole PBMCs (Figure 1c). Surface CD46 expression in T cells was more largely reduced than that in B cells. We also investigated seven additional human cells (Molt-4, KG-1a, K562, U937, A549, HeLa and human bone marrow-derived $CD34^+$ cells) for Ad35 vector-induced downregulation of surface CD46 levels; the downregulation levels of surface CD46 were different among the cell types (Table 1). K562, U937, KG-1a and Molt-4 cells exhibited a decrease in CD46 expression following Ad35 vector infection (by 36% in K562 cells, 24% in U937 cells, 18% in KG-1a cells

Table 1 Downregulation of CD46 induced by Ad35 vectors in various types of cells

Cell type	*% CD46 downregulation*
Molt-4	55±5.7
KG-1a	18±2.6
K562	36±1.9
U937	24±8.6
A549	−10±8.0
HeLa	7.9±18
Human bone marrow-derived $CD34^+$ cells	−11±5.2

The cells were infected with Ad35L at 10 000 VP/cell. After incubation for 24 h, CD46 expression on the cell surface was determined by flow cytometry as described in Materials and methods. Values represent mean±s.d. of quadruplicate results from two similar experiments.

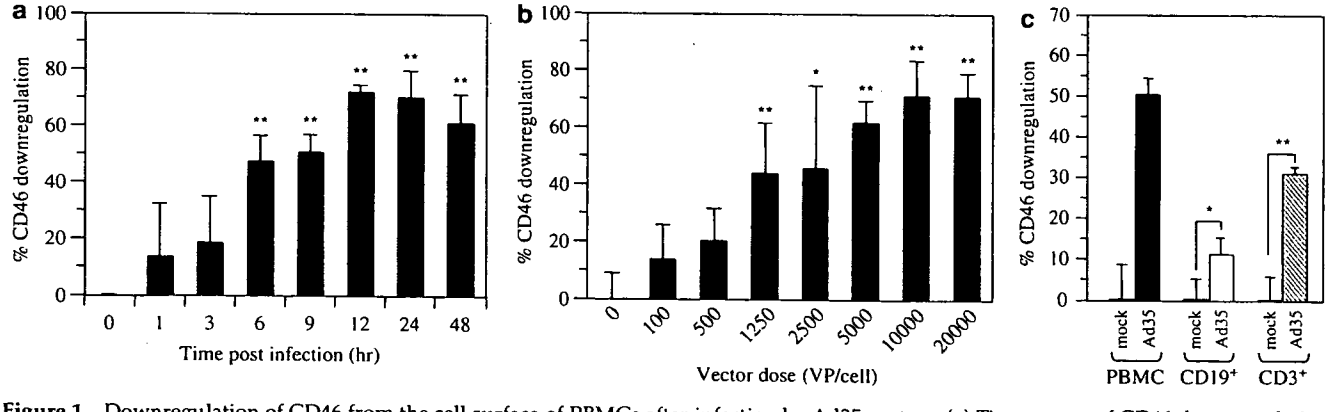

Figure 1 Downregulation of CD46 from the cell surface of PBMCs after infection by Ad35 vectors. (a) Time course of CD46 downregulation from the cell surface of PBMCs after infection with Ad35GFP. PBMCs were incubated with Ad35GFP at 10 000 VP/cell for up to 48 h. Cells were harvested at the indicated time points and stained with anti-human CD46 antibodies after fixation. The expression levels of CD46 on the cell surface were determined by flow cytometry. (b) Dose-dependent downregulation of surface CD46 after infection with Ad35 vectors. PBMCs were infected with Ad35GFP at the indicated vector doses for 24 h. After incubation for 24 h, PBMCs were harvested and CD46 expression levels were determined by flow cytometry. (c) Surface CD46 downregulation in B cells and T cells after infection with Ad35 vectors. PBMCs were infected with Ad35L at 10 000 VP/cell. After incubation for 24 h, PBMCs were harvested and stained with both anti-human CD46 antibody and anti-human CD19 or anti-human CD3 antibody. Subsequently, the cells were subjected to flow cytometric analysis. The asterisks indicate the level of significance ($P<0.005$ (double asterisk), $P<0.05$, (single asterisk)). Values represent mean±s.d. of quadruplicate results from one of at least two similar experiments.

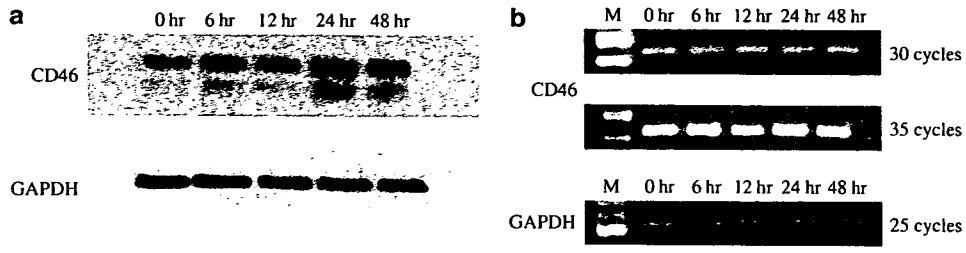

Figure 2 Total cellular protein levels and mRNA levels of CD46 following infection by Ad35 vectors. (a) Western blotting analysis for the total cellular protein levels of CD46 in PBMCs infected with Ad35GFP. PBMCs were incubated with Ad35GFP at 10 000 VP/cell for up to 48 h. Cells collected at the indicated time points were lysed and quantified by immunoblotting for their total cellular amounts of CD46. GAPDH bands served as an internal control for equal total protein loading. This result was representative of three independent experiments. (b) Semiquantitative RT-PCR analysis of CD46 in PBMCs infected with Ad35GFP. PBMCs were infected with Ad35GFP as described for Western blotting analysis in Materials and methods. Total RNA was prepared from PBMCs following incubation with Ad35GFP, and RT-PCR was then performed as described in Materials and methods. Lane M: 100-bp ladder. These results were representative of at least two independent experiments.

and 55% in Molt-4 cells), whereas CD46 expression was reduced not at all or only slightly in nonleukemia cells (A549, HeLa and bone marrow CD34$^+$ cells). Indeed, a slight increase in CD46 expression on the cell surface was found in A549 and CD34$^+$ cells following Ad35 vector infection.

Total protein levels and mRNA levels of CD46 are not reduced following Ad35 vector infection

To examine the mechanism of Ad35 vector-induced downregulation of surface CD46, Western blotting and semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for CD46 expression were performed. Western blotting analysis using total cellular lysates demonstrated that the total cellular levels of CD46 were not reduced, but rather seemed to slightly increase, during 48 h of exposure to Ad35GFP (Figure 2a), suggesting that CD46 may be internalized after infection by Ad35 vectors without intracellular degradation, as in the case of MV.[7] In addition, infection by Ad35GFP did not decrease the mRNA levels of CD46 (Figure 2b). These results indicate that infection by Ad35 vectors does not downregulate the transcription of the *CD46* gene.

Fiber knob proteins of subgroup B Ads play a crucial role in the decrease in surface CD46 expression

To investigate which parts of Ad35 are involved in the downregulation of surface CD46 levels, PBMCs were incubated with conventional Ad5 vectors, fiber-substituted Ad5 vectors displaying the Ad35 fiber shaft and knob, and ultraviolet (UV)- or heat-inactivated Ad35 vectors at 10 000 VP/cell for 24 h. The conventional Ad5 vectors expressing GFP, Ad5GFP, which utilizes CAR for infection, did not downregulate CD46, whereas infection by the Ad5F35 vector expressing GFP, Ad5F35GFP, which recognizes CD46 for infection, significantly reduced surface CD46 expression by 72% (Figure 3a). UV-inactivated Ad35GFP also induced the downregulation of surface CD46 by 62% following infection, which was a level similar to that of surface CD46 downregulation induced by Ad35GFP. However, heat-inactivated Ad35GFP produced a lower level of surface CD46 downregulation than UV-inactivated Ad35GFP. This low level of CD46 downregulation by heat-inactivated Ad35GFP

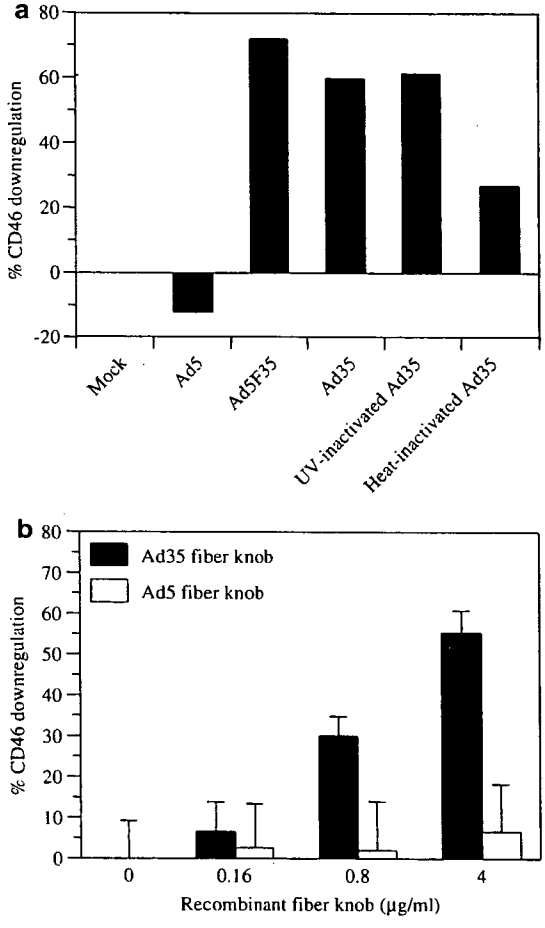

Figure 3 Role of Ad35 fiber knob protein on downregulation of surface CD46. (a) Downregulation of surface CD46 expression induced by various types of Ad vectors. PBMCs were incubated with Ad5GFP, Ad5F35GFP, Ad35GFP, UV-inactivated Ad35GFP or heat-inactivated Ad35GFP at 10 000 VP/cell for 24 h. After incubation, the cells were subjected to flow cytometric analysis to determine the level of CD46 expression. Values represent the mean of duplicate results from one of three similar experiments. (b) Downregulation of surface CD46 expression induced by Ad35 fiber knob protein. PBMCs were incubated with Ad5 or Ad35 fiber proteins at the indicated concentrations. After incubation for 24 h, the cells were subjected to flow cytometric analysis for the measurement of surface CD46 levels. Values represent the mean of quadruplicate results from one of three similar experiments.

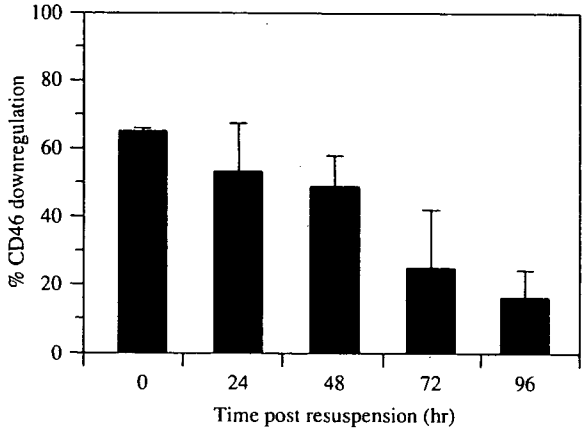

Figure 4 Recovery of surface CD46 expression after Ad35 vector-mediated downregulation. PBMCs were infected with Ad35GFP at 10 000 VP/cell for 24 h. After a 24-h infection, PBMCs were washed twice to remove the Ad35GFP, and resuspended and incubated in fresh medium. After incubation, cells were harvested at the indicated time points and CD46 expression was measured by flow cytometry. Values represent mean ± s.d. of quadruplicate results from one of two similar experiments.

would be due to the thermal denaturation of fiber proteins in Ad35GFP.

Next, PBMCs were incubated with recombinant Ad5 or Ad35 fiber knob proteins to further examine the role of Ad35 fiber protein in the downregulation of surface CD46 expression. Ad35 fiber knob proteins were found to downregulate the surface expression levels of CD46 in a dose-dependent manner, and maximal downregulation of surface CD46 was induced by 55% at 4 μg/ml (Figure 3b). In contrast, no significant reduction in surface CD46 levels was found after incubation with Ad5 fiber knob proteins. These results indicate that fiber knob proteins of Ad35 play a crucial role in the downregulation of surface levels of CD46.

Downregulated CD46 expression is not rapidly restored after the removal of Ad35 vectors

Next, we examined how long it takes to restore surface CD46 expression after Ad35 vector-induced downregulation. Downregulation of surface CD46 induced by Ad35GFP gradually recovered after resuspension, however, the recovery kinetics of CD46 expression after the removal of Ad35 vectors was much lower than the kinetics of Ad35 vector-induced decrease in the surface CD46 (Figure 4). CD46 expression was downregulated by 65% before resuspension, and surface CD46 expression remained reduced by 53 and 49% at 24 and 48 h after resuspension, respectively. Complete restoration of surface CD46 expression was not observed even at 96 h after resuspension, at which point 17% downregulation remained, thus, more than 96 h are required to restore completely surface CD46 expression after Ad35 vector-induced downregulation.

Discussion

Understanding the interaction between cellular receptors and viruses and subsequent events following the attachment of viruses to receptors is important to elucidate the tropism, infectivity and pathogenicity of viruses. Many previous studies have assessed the interaction between CD46 and CD46-utilizing pathogens, especially MV, and have demonstrated that infection by CD46-utilizing pathogens causes unique cellular events. For example, downregulation of CD46 from the cell surface occurs following infection by MV,[7,17] HHV6[4] or *Neisseria*.[9] Additionally, MV and HHV6 suppress interleukin (IL)-12 production in infected human monocytes.[29,30] However, subsequent cellular events following the interaction between human CD46 and subgroup B Ads have not yet been fully evaluated. In the present study, we have demonstrated the downregulation of human CD46 from the cell surface following infection by Ad35 vectors belonging to subgroup B.

MV-induced downregulation of surface CD46 has been the most thoroughly studied aspect of the effects of pathogens recognizing CD46. Nevertheless, the precise mechanisms of MV-induced downregulation of surface CD46 remain to be clarified; surface CD46 downregulation by MV exhibits similar properties to that induced by Ad35 vectors. First, surface expression levels of CD46 are reduced, whereas the total cellular protein levels of CD46 are not significantly decreased after infection,[7] as demonstrated by Western blotting analysis (Figure 2a). These results suggest that CD46 may be internalized without degradation following infection by MV or Ad35. Second, the protein components, which bind to CD46 in the virion, MVH proteins and fiber knob proteins of Ad35, are involved with surface CD46 downregulation. Previous studies indicate that direct protein–protein contact between CD46 and MVH proteins is necessary for the MV-induced downregulation of surface CD46 levels.[31,32] The present data in Figure 3 indicate that fiber knob proteins of Ad35 play a crucial role in the reduction in surface CD46 expression. These common properties suggest that Ad35 might downregulate the surface expression levels of CD46 through a mechanism similar to the one that acts in the case of MV. This hypothesis is further supported by previous findings that both the MVH and fiber knob proteins of Ad35 recognize the domains within SCR1 and 2 of CD46.[13–15,33,34]

However, the Ad35 vector-mediated modulation of CD46 expression in nonleukemia cells differed from that induced by MVH protein: Ad35 vectors did not produce any decline in CD46 expression in the nonleukemia cells used in the present study (HeLa, A549 and human bone marrow-derived $CD34^+$ cells). We have also demonstrated that surface CD46 expression was not decreased following Ad35 vector infection in Chinese hamster ovary (CHO) transformants expressing CD46[15] (data not shown), however, the MVH protein downregulated CD46 expression in HeLa cells[31] and in CHO transformants stably expressing CD46.[35] These findings suggest that cellular events following the binding of Ad35 vectors to CD46 would be somewhat different from those induced by MV in nonleukemia cells.

Downregulation of surface CD46 levels by Ad35 vectors seems inefficient compared with that induced by MV. An approximately 24% reduction in CD46 expression was achieved in U937 cells 24 h following infection of Ad35L at 10 000 VP/cell, which is an approximate multiplicity of infection (MOI) of 50. In contrast, infection by MV strain Edmonston in U937 cells

induced a decline of about 70% in CD46 expression 12 h after infection even at an MOI of 5.[31] The lower levels of surface CD46 downregulation caused by replication-incompetent Ad35 vectors might be partly due to a lack of virus replication; a previous study suggests that newly synthesized MVH proteins in the infected cells further downregulate surface CD46 expression.[31]

Piliated *N. gonorrhoeae*, which also utilizes CD46 as a receptor, exhibits surface CD46 downregulation by the shedding of CD46.[9] The total levels of CD46 in the whole-cell lysates are reduced, and soluble CD46 is found in cell culture supernatants after exposure to piliated *N. gonorrhoeae*. It now remains unclear how piliated *N. gonorrhoeae* induces shedding of CD46. However, it is unlikely that Ad35 vector-induced shedding of CD46 occurs because total cellular levels of CD46 were not reduced following infection with Ad35 vectors (Figure 2a).

It is surprising that the downregulation of surface CD46 expression was not readily restored after the removal of Ad35 vectors because pulse-chase analysis of CD46 showed that matured forms of CD46 are synthesized within 1 h.[36] This raises the question of why newly synthesized CD46 is not transported to the surface membrane in Ad35 vector-infected cells and where newly synthesized CD46 stays in the cells. Further studies are necessary to address these questions.

Previous studies have demonstrated the increased susceptibility of cells to complement-mediated lysis as a result of surface CD46 downregulation,[10,31] however, we found no apparent lysis of PBMCs *in vitro* by complements following Ad35 vector infection (data not shown). It is now unclear why the complement-mediated cell lysis did not occur in cells showing CD46 downregulation by Ad35 vectors. One possible explanation is that the decreased levels of surface CD46 by Ad35 vectors might be enough to block the complement-mediated cell lysis. Other complement regulatory proteins might compensate the reduction in surface CD46 levels. Although PBMCs showing the reduction in surface CD46 density did not exhibit an apparent increase in susceptibility to complement-mediated cell lysis *in vitro*, this study suggests that we should exercise caution in the use of CD46-utilizing Ad vectors. The reduction in CD46 expression in cells transduced with CD46-utilizing Ad vectors might cause unexpected side effects after *in vivo* application. Recently, CD46 has been demonstrated to be involved in not only complement regulation but also various cellular functions, such as immune responses.[37,38] It is essential to further examine the influence of surface CD46 downregulation, including the fate of the transduced cells, before initiating clinical applications of CD46-utilizing Ad vectors. Additionally, the influence of surface CD46 downregulation *in vivo* should be evaluated in nonhuman primates; the use of human CD46-transgenic mice is not recommended because rodent CD46 expression is limited in testis, and other complement regulators, such as decay-accelerating factor, protect cells from complement attack in rodents.[39]

In summary, we have shown that infection by Ad35 vectors induces downregulation of human CD46 from the cell surface in a dose-dependent and cell type-specific manner. In addition to Ad35 vectors, fiber-substituted Ad5 vectors containing fiber proteins derived from Ad35 also downregulate the surface expression of CD46. Once the surface expression levels of CD46 have declined, CD46 expression is not readily restored after the removal of Ad35 vectors. The present study provides important clues for clarifying the pathogenicity of subgroup B Ad, and suggests caution in the use of Ad vectors recognizing CD46 for gene therapy.

Materials and methods

Cells

Human PBMCs (Cambrex Bio Science, Walkersville, MD, USA) were cultured in culture medium (Roswell Park Memorial Institute (RPMI)1640 supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 4 mM L-glutamine, 10% fetal bovine serum (FBS)). HeLa cells (human cervix epitheloid carcinoma) were cultured with Dulbecco's modified Eagle's medium supplemented with 10% FBS. A549 cells (a human lung epithelial cell line) were cultured with F-12K medium supplemented with 10% FBS. K562 cells (human chronic myelogenous leukemia in blast crisis), U937 cells (a human lymphoma cell line), Molt-4 (a human T-cell leukemia cell line) and KG-1a cells (human bone marrow acute myelogenous leukemia) were cultured with RPMI1640 medium supplemented with 10% FBS. Human bone marrow-derived $CD34^+$ cells (Cambrex Bio Science) were cultured with StemSpan 2000 containing cytokine cocktail StemSpan CC100 (human Flt-3 ligand (100 ng/ml), human stem cell factor (100 ng/ml), human IL-3 (20 ng/ml) and human IL-6 (20 ng/ml)) (StemCell Technologies Inc., Vancouver, BC, Canada).

Ad vectors

Ad35 vectors containing a cytomegalovirus promoter-driven enhanced GFP expression cassette or a cytomegalovirus promoter-driven firefly luciferase expression cassette, Ad35GFP and Ad35L, respectively, were constructed by the improved *in vitro* ligation method described previously.[40] GFP-expressing conventional Ad5-based vectors, Ad5GFP and fiber-substituted Ad5-based vectors displaying the fiber knob and shaft of Ad35, Ad5F35GFP, were also constructed as described previously.[25,41] Determination of the virus particle titers of Ad vectors was accomplished following the method described by Maizel *et al.*[42] Ad35GFP was UV- and heat-inactivated by exposure to 254-nm radiation for 1 h, and by incubation at 48°C for 1 h, respectively. The efficiency of the inactivation was confirmed by comparing the transduction efficiencies of control and inactivated Ad35GFP.

Downregulation of surface CD46 by infection with Ad35 vectors

For the present time course study of the downregulation of surface CD46, PBMCs were seeded in a 96-well plate at 5.0×10^4 cells/well and incubated with Ad35GFP at 10 000 VP/cell. PBMCs were harvested at various time points and subjected to flow cytometric analyses as described below. For the study of the dose-dependent downregulation of surface CD46, PBMCs were infected with Ad35GFP at the indicated vector doses. After incubation for 24 h, the surface expression levels of CD46

were measured by flow cytometry. Analysis of the downregulation of surface CD46 levels in response to various types of Ad vectors was similarly performed. Ad35 vector-mediated decrease in the surface CD46 levels was also assessed in various types of human cells (Molt-4, KG-1a, K562, U937, A549, HeLa and human bone marrow-derived $CD34^+$ cells). Cells were seeded in a 24- or 96-well plate and infected with Ad35L at 10 000 VP/cell. After incubation for 24 h, CD46 expression levels were assessed by flow cytometry.

Ad35 fiber knob-mediated downregulation of surface CD46

Recombinant Ad35 fiber knob protein was constructed similarly to Ad5 fiber knob,[43] using Ad35 vector plasmid pAdMS4[44] and the following primers: forward, 5′-tcg aat tca cct tat gga ctg gaa taa acc c-3′ (*Eco*RI site is underlined); reverse, 5′-atg cgg ccg ctt agt tgt cgt ctt ctg taa tgt aag a-3′ (*Not*I site is underlined). Ad5 fiber knob protein was prepared previously.[43] PBMCs, which were seeded in a 96-well plate at 5.0×10^4 cells/well, were incubated with the Ad5 or Ad35 fiber knob at the indicated concentrations. Surface CD46 expression levels were examined 24 h after incubation by flow cytometry as described below.

Western blotting analysis for CD46 expression

PBMCs (5.0×10^5 cells) were seeded in a 24-well plate and infected with Ad35GFP at 10 000 VP/cell. They were then collected at the indicated time points, washed and treated with lysis buffer (25 mM Tris, 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM ethylenediaminetetraacetic acid, 150 mM NaCl) containing a cocktail of protease inhibitors (Sigma, St Louis, MO, USA). The protein content in the cell lysates was measured with an assay kit from Bio-Rad (Hercules, CA, USA), using bovine serum albumin (BSA) as a standard. Protein samples (10 μg) were subjected to nonreducing sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis, and the separated proteins were transferred to a nitrocellulose membrane. After blocking nonspecific binding, CD46 was detected with anti-CD46 rabbit serum (1:5000; kindly provided by Dr Tsukasa Seya, Hokkaido University, Japan), followed by incubation in the presence of horseradish peroxidase-labeled goat anti-rabbit second antibody (1:6000, Cell Signaling, Danvers, MA, USA). Signals on the membrane were visualized and analyzed as described previously.[40] To verify equal loading, the blots were stripped and probed with a rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:3000, Trevigen, Gaithersburg, MD, USA) followed by treatment with an horseradish peroxidase-conjugated goat anti-rabbit second antibody (1:5000, Cell Signaling).

RT-PCR analysis for CD46 expression

PBMCs were infected with Ad35GFP as performed in Western blotting analysis. After infection, the cells were collected at the indicated time points and total RNA was isolated from the cells using Isogen reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA templates were synthesized as previously described,[43] and the templates were subjected to PCR amplification using sets of primers for human CD46[45] and GAPDH.[46] The cycling parameters were 30 s at 94°C, 30 s at 55°C and 30 s at 72°C for both CD46 and GAPDH. PCR products were separated by electrophoresis on a 2.0% agarose gel and visualized with ethidium bromide.

Recovery of CD46 expression from the Ad35 vector-mediated downregulation of surface CD46 expression

PBMCs seeded in a six-well plate were infected with Ad35GFP at 10 000 VP/cell. After a 24-h incubation, the cells were collected and washed twice to remove the Ad35GFP. The PBMCs were then resuspended in fresh culture medium, and subsequently cultured at 37°C PBMCs were harvested at the indicated time points and subjected to flow cytometric analysis to measure CD46 expression.

Flow cytometry

Cells were harvested, washed with FACS buffer (phosphate-buffered saline (PBS) containing 1% BSA and 0.01% sodium azide) and then fixed for 10 min with 3.2% paraformaldehyde-containing PBS. Cells were washed twice and incubated with anti-human CD46 antibody (J4.48, Immunotech, Marseilles, France; or E4.3, Pharmigen, San Diego, CA, USA) for 45 min on ice. Subsequently, the cells were washed and incubated with phycoerythrin (PE)-conjugated goat anti-mouse IgG second antibody (Pharmingen). After being washed thoroughly, stained cells were analyzed by FACSCalibur (Becton Dickinson, Tokyo, Japan) and CellQuest software (Becton Dickinson) to obtain the percentage of surface CD46 downregulation as follows: CD46 downregulation = 100−(100 × MFI of CD46 in infected cells)/(MFI of CD46 in uninfected cells), where MFI = mean fluorescence intensity.

For the simultaneous analysis of expression levels of CD46 and CD19 (B-cell marker) or CD3 (T cell marker), PBMCs were incubated with both fluorescein isothiocyanate (FITC)-labeled anti-human CD46 antibody (E4.3, Pharmingen) and PE-conjugated anti-human CD19 antibody (HIB19, Pharmingen) or allophycocyanin (APC)-labeled anti-human CD3 antibody (UCHT1, eBioscience, San Diego, CA, USA). After incubation for 45 min on ice, stained cells were subjected to flow cytometry analysis as described above.

Abbreviations

Ad, adenovirus; APC, allophycocyanin; BSA, bovine serum albumin; CAR, coxsackievirus and adenovirus receptor; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescence protein; HHV6, herpesvirus type 6; MV, measles virus; MVH, measles virus hemagglutinin; MFI, mean fluorescence intensity; MOI, multiplicity of infection; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; SCRs, short consensus repeats; VP, vector particle; PE, phycoerythrin.

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ORIGINAL ARTICLE

Interaction of penton base Arg-Gly-Asp motifs with integrins is crucial for adenovirus serotype 35 vector transduction in human hematopoietic cells

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Most subgroup B adenoviruses (Ads), including adenovirus (Ad) serotype 35 (Ad35), bind to human CD46 as a receptor; however, the infection processes of subgroup B Ads following attachment to CD46 remain to be elucidated. Subgroup B Ads possess Arg-Gly-Asp (RGD) motifs in the penton base, similarly to subgroup C Ad serotypes 2 and 5. In this study, we examined the role of penton base RGD motifs in Ad35 vector-mediated transduction in human hematopoietic cells. Inhibition of interaction between integrins and the RGD motifs by divalent cation chelation and a synthetic RGD peptide reduced the transduction efficiencies of Ad35 vectors; however, the amounts of cell-associated vector DNA of Ad35 vectors at 4 or 37°C were not decreased by divalent cation chelation or the RGD peptide. Mutation of penton base RGD motifs reduced the transduction efficiencies of Ad35 vectors, although the amounts of cell-associated vector DNA of Ad35 vectors at 4 or 37°C were not altered by mutation of penton base RGD motifs in Ad35 vectors. Furthermore, preincubation with several types of anti-integrin antibodies significantly inhibited Ad35 vector-mediated transduction. These results suggest that interaction between integrins and penton base RGD motifs plays a crucial role in Ad35 vector-mediated transduction in hematopoietic cells, probably in the post-internalization steps.
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Keywords: *adenovirus serotype 35; penton base; integrins; RGD motif; hematopoietic*

Introduction

Human adenoviruses (Ads) are nonenveloped, double-stranded DNA viruses currently containing 51 serotypes, which are classified into six distinct subgroups (A–F).[1] Among these serotypes, adenovirus (Ad) vectors based on Ad serotype 5 (Ad5), which belongs to subgroup C, are the most commonly used for gene therapy and have been the most extensively studied, including studies of their transduction mechanisms. As the first step for the entry of Ad5 vectors into cells, the fiber knob binds to the primary receptor, coxsackievirus-adenovirus receptor (CAR). After binding to CAR, Arg-Gly-Asp (RGD) motifs located in the penton base interact with cellular integrins, including $\alpha_v\beta_1$,[2] $\alpha_v\beta_3$, $\alpha_v\beta_5$,[3,4] $\alpha_5\beta_1$,[5] and $\alpha_M\beta_2$,[6] leading to the internalization of Ad5 vectors via clathrin-coated pits. In these transduction processes, binding to CAR is the most crucial determinant of the transduction efficiency of Ad5 vectors, and Ad5 vector-mediated gene transfer to cells lacking CAR expression is inefficient. Regrettably, several important target cells for gene therapy, including hematopoietic stem cells,[7] dendritic cells[8] and malignant tumor cells,[9,10] express low or negligible levels of CAR.

On the other hand, most subgroup B Ads, including Ad serotype 11 and 35 (Ad35), attach to human CD46 as a receptor.[11,12] Human CD46 is ubiquitously expressed in almost all human cells, leading to the broad tropism of subgroup B Ad vectors in human cells, one of the subgroup's advantages. In particular, hematopoietic cells, which are important targets for gene therapy but resistant to Ad5 vectors, are susceptible to subgroup B Ads.[13–15] Infection of subgroup B Ads is initiated by the attachment of the fiber knob to short consensus repeats 1 and 2 of human CD46.[16–18] However, the infection process of subgroup B Ads following binding to CD46 is poorly understood. Elucidation of the transduction mechanism would lead to more effective applications and improvements of subgroup B Ad vectors. Previous studies demonstrated that subgroup B Ads possess an RGD motif in the penton base similarly to Ad5,[19–21] and that CD46 is associated with some of the integrins on the cell surface,[22] which caused us to hypothesize that interaction between penton base RGD motifs and integrins plays an important role in subgroup B Ad vector-mediated transduction.

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In this study, we examined the involvement of interaction between integrins and penton base RGD motifs in subgroup B Ad35 vector-mediated transduction in human bone marrow-derived CD34$^+$ cells and human leukemia cell line K562 cells, both of which have been shown to be highly susceptible to Ad35 vectors.[15,23,24] Human bone marrow-derived CD34$^+$ cells are an important target for gene therapy, because the CD34$^+$ cells are a fraction that contains hematopoietic stem cells. We found that inhibition of the interaction between integrins and penton base RGD motifs did not alter total amounts of cell-associated vector DNA of Ad35 vectors at 4 or 37 °C; however, it significantly reduced the transduction efficiencies of Ad35 vectors, suggesting that interaction between integrins and penton base RGD motifs is largely involved with Ad35 vector-mediated transduction, probably in postinternalization steps.

Results

Several types of integrins are expressed on human bone marrow-derived CD34$^+$ cells and K562 cells

First, we performed flow cytometric analysis to examine which types of integrins are expressed on human bone marrow-derived CD34$^+$ cells and K562 cells. Flowcytometric analysis demonstrated that the CD34$^+$ cells and K562 cells both expressed high levels of integrins α_5 (95%; CD34$^+$ cells, 100%; K562 cells) and β_2 (99%; CD34$^+$ cells, 86%; K562 cells) (Figure 1). Integrins α_4, α_6 and β_1 were also expressed in the CD34$^+$ cells, but hardly detected in K562 cells. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which are well known as second receptors for Ad5 vectors,[3,4] were expressed in low levels in both cells ($\alpha_v\beta_3$; 13 and 20% in CD34$^+$ cells and K562 cells, respectively; $\alpha_v\beta_5$; 2 and 17% in CD34$^+$ cells and K562 cells, respectively).

Divalent cations are required for Ad35 vector-mediated transduction

Next, to examine involvement of integrins in Ad35 vector-mediated transduction, the hematopoietic cells were transduced with Ad35 vectors in the presence of EDTA or divalent cations. The affinity between integrins and their ligands is regulated by divalent cations.[25–27] Notably, Mn^{2+} is reported to largely promote the binding of ligands to integrins. Transduction efficiencies of an Ad35 vector expressing an enhanced green fluorescence protein (GFP) (Ad35GFP) in K562 cells were significantly reduced in the presence of EDTA (Figure 2a). Treatment with 5 mM EDTA decreased GFP expression by 80%. On the other hand, Mn^{2+} significantly increased the transduction efficiencies of Ad35GFP in K562 cells, while exposure to Mg^{2+} or Ca^{2+} did not affect the transduction efficiencies of Ad35GFP (Figure 2b). These results suggest that integrins are involved with Ad35 vector infection. Transduction experiments using the CD34$^+$ cells were also tried; however, pretreatments with EDTA or divalent cations largely reduced the viability of the CD34$^+$ cells (data not shown).

To investigate the mechanisms of EDTA-mediated decrease and the Mn^{2+}-mediated increase in transduction efficiencies of Ad35 vectors, cellular binding and uptake of Ad35GFP in the presence of EDTA or Mn^{2+} was assessed by real-time PCR. Exposure to 5 mM EDTA or Mn^{2+} did not increase the total amounts of Ad35 vector genome associated with K562 cells at 4 °C 1.5 h following infection, indicating that cellular binding of Ad35GFP is not affected by divalent cations (Figure 3). In addition, the cells treated with EDTA or Mn^{2+} also showed no change in the amounts of cell-associated Ad35 vector DNA at 37 °C (the sum of cellular binding and uptake of Ad35GFP). These results suggest that postinternalization steps in Ad35 vector-mediated transduction are affected by EDTA and Mn^{2+}.

Penton base RGD motifs are involved with Ad35 vector-mediated transduction

To examine whether the RGD motifs in the Ad35 penton base are involved with Ad35 vector-mediated transduction in the CD34$^+$ cells and K562 cells, the cells were transduced with Ad35GFP in the presence of synthetic RGD peptide. Transduction with Ad35GFP was significantly suppressed by the RGD peptide in a dose-dependent fashion in the human CD34$^+$ cells and K562 cells (Figure 4). The GFP expression levels by Ad35GFP in the CD34$^+$ cells and K562 cells were respectively

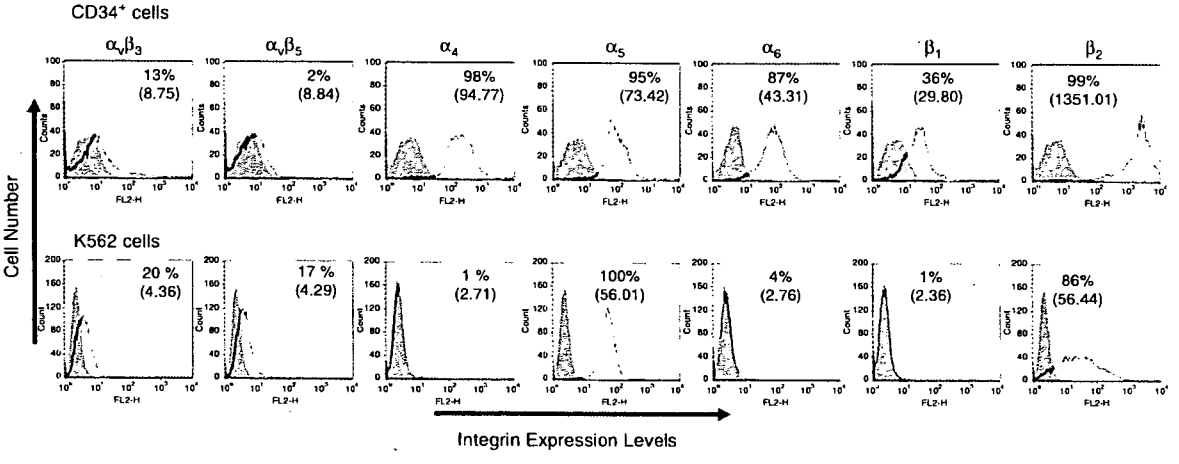

Figure 1 Flow cytometric profiles of integrin expressions on human bone marrow-derived CD34$^+$ cells and K562 cells. The cells were stained with monoclonal anti-integrin antibodies, followed by a phycoerythrin-labeled secondary antibody, and subsequently analyzed by a flow cytometry (thick line). As a negative control, cells were incubated with an isotype control antibody (shaded histogram). Percentages of positive cells (and mean fluorescence intensities) are shown by number in the upper right-hand corner of each profile.

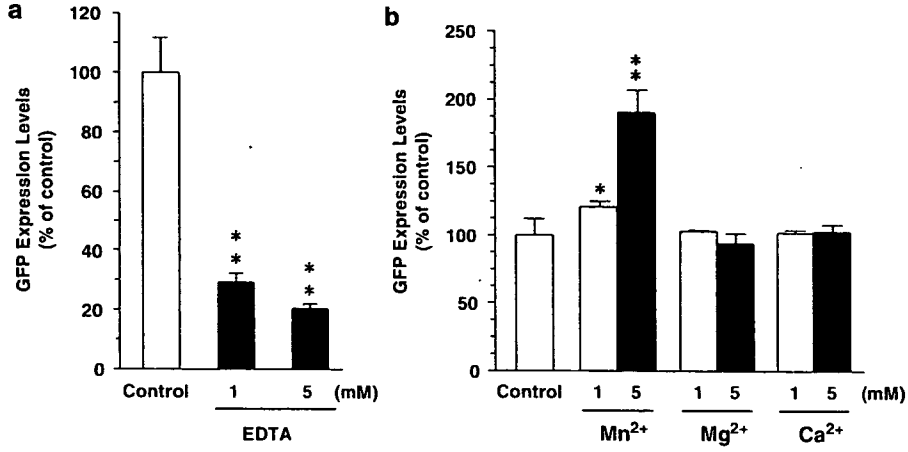

Figure 2 Effects of divalent cations on Ad35 vector-mediated transduction. (a) Ad35GFP-mediated transduction in the presence of EDTA. K562 cells were transduced with Ad35GFP at 1000 VP per cell for 1.5 h in the presence of EDTA at the indicated concentrations. GFP expression levels were measured using flow cytometry 48 h after transduction. The GFP expression levels (mean fluorescence intensity; MFI) in the absence of EDTA (control) and the mock-infected cells were 107.6 and 2.4, respectively. (b) Ad35GFP-mediated transduction in the presence of Mn^{2+}, Mg^{2+} or Ca^{2+} ions. K562 cells were preincubated in TBS buffers containing $MnCl_2$, $MgCl_2$ or $CaCl_2$ at indicated concentrations for 30 min and then transduced with Ad35GFP at 1000 VP per cell. The transduction experiments were performed as described above. The GFP expression level (MFI) in TBS buffer (control) and the mock-infected cells were 119.3 and 2.3, respectively. The data were normalized to the GFP expression levels (MFI) in K562 cells, which were preincubated in TBS buffer prior to transduction (control). Data are expressed as means ± s.d. ($n = 3$ or 4). $^*P < 0.05$, $^{**}P < 0.001$ in comparison with the control. TBS, Tris-buffered saline.

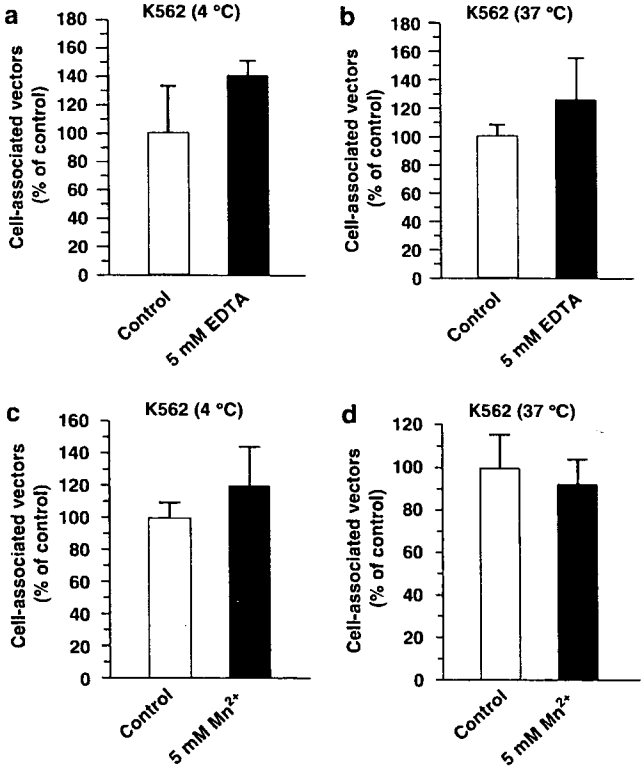

Figure 3 Effects of divalent cations on cellular binding and uptake of Ad35 vectors in K562 cells. K562 cells were incubated with Ad35GFP in the presence of 5 mM EDTA (**a**, **b**) or 5 mM Mn^{2+} (**c**, **d**) as described in Figure 2. After incubation, the cells were washed and total DNA, including Ad vector DNA, was recovered. The vector copy number was quantified using real-time PCR. The data were normalized to the vector copy number when transduced with Ad35GFP in Tris-buffered saline buffer. Data are expressed as means ± s.d. ($n = 4$). GFP, green fluorescence protein.

decreased by 57 and 24% in the presence of 200 μg ml^{-1} of the RGD peptide, compared with the presence of the control RGE peptide.

Next, to clarify why coincubation with the RGD peptide decreases the transduction efficiencies of Ad35 vectors, we evaluated cellular binding and uptake of Ad35 vectors in the presence of the RGD peptide. As shown in Figure 5, the RGD peptide did not decrease the total amounts of cell-associated vector DNA of Ad35GFP at 4 or 37 °C in the $CD34^+$ cells and K562 cells, suggesting that the interaction between integrins and penton base RGD motifs are crucial for Ad35 vector-mediated transduction; however, this interaction would not be largely involved with the cellular binding or uptake of Ad35 vectors in the $CD34^+$ cells and K562 cells.

Mutation of penton RGD motifs in Ad35 vectors significantly reduces the transduction activity of Ad35 vectors

To further examine the involvement of penton RGD motifs with Ad35 vector-mediated transduction, we constructed Ad35 vectors containing the RGE sequence instead of RGD in the penton base (D343E) (Ad35RGE-GFP) or a deletion of the RGD motifs in the penton base (Ad35ΔRGD-GFP) (Table 1). Final yields of Ad35RGE-GFP and Ad35ΔRGD-GFP were comparable to those of Ad35GFP (data not shown). The $CD34^+$ cells exhibited approximately 80% reduction in GFP expression following transduction with Ad35RGE-GFP and Ad35ΔRGD-GFP at 6000 vector particles (VP) per cell (Figure 6a). In K562 cells, Ad35RGE-GFP and Ad35ΔRGD-GFP mediated approximately 35% reduced GFP expression compared with Ad35GFP at 3000 VP per cell (Figure 6b). Transduction with each Ad35 vector at lower doses resulted in similar transduction profiles to those shown here in the $CD34^+$ cells and K562 cells (data not shown),

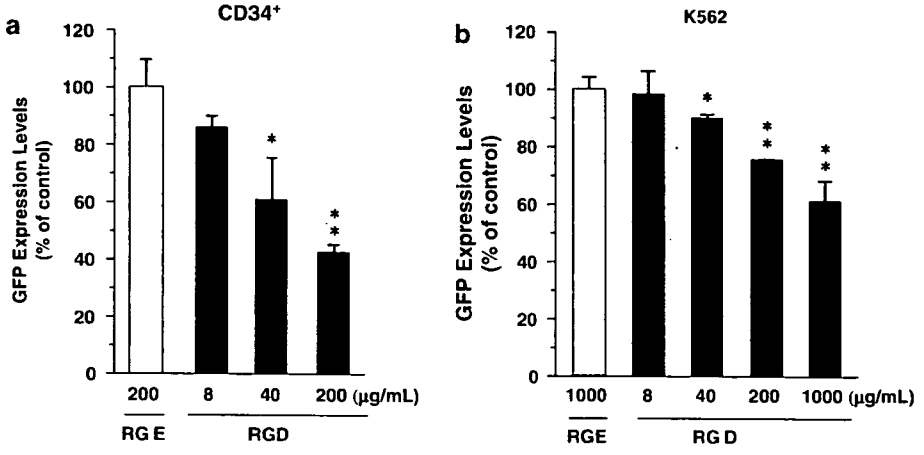

Figure 4 Inhibition of Ad35 vector-mediated transduction by synthetic RGD peptide. Human CD34+ cells (**a**) and K562 cells (**b**) were transduced with Ad35GFP at 3000 VP per cell for 3 h (human CD34+ cells) or at 1000 VP per cell for 1 h (K562 cells) at 37 °C in the presence of RGD peptides. GFP expression levels were measured 48 h after transduction by flow cytometry. Data were normalized to the GFP expression levels (MFI) in the presence of control RGE peptide. The GFP expression levels (MFI) in the presence of control RGE peptide (CD34+ cells; 200 μg ml^{-1}, K562 cells; 1000 μg ml^{-1}) were 198.2 (CD34+ cells) and 748.9 (K562 cells). The GFP expression levels (MFI) in the mock-infected cells were 2.3 (CD34+ cells) and 2.9 (K562 cells). The Data are expressed as means ± s.d. ($n = 3$). $^*P < 0.05$, $^{**}P < 0.001$ in comparison with control RGE peptide. MFI, mean fluorescence intensity; RGD, Arg-Gly-Asp.

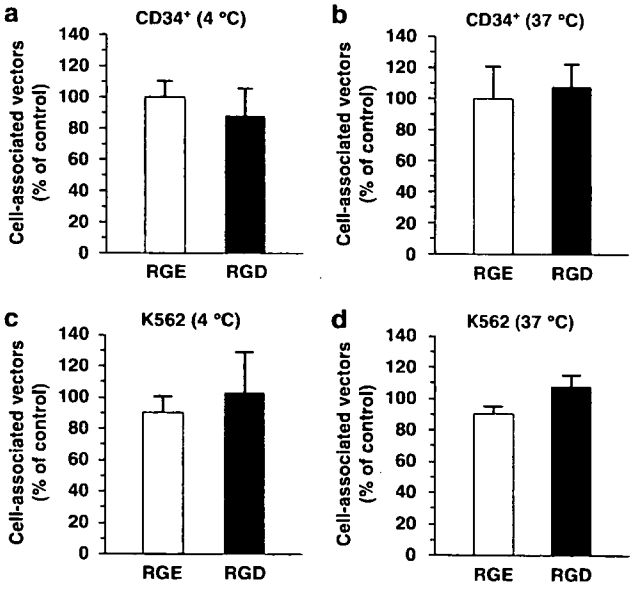

Figure 5 Cellular binding and uptake of Ad35GFP in the presence of RGD peptide. Human CD34+ cells (**a**, **b**) and K562 cells (**c**, **d**) were incubated with Ad35GFP in the presence of RGD peptide (200 μg ml^{-1}) as described in Figure 4. After incubation, the cells were washed, and total DNA, including Ad vector DNA, was recovered. The vector copy number was quantified using real-time PCR. The data were normalized to the vector copy number of Ad35GFP in the presence of control RGE peptide. Data are expressed as mean ± s.d. ($n = 4$). GFP, green fluorescence protein; RGD, Arg-Gly-Asp; RGE, Arg-Gly-Glu.

suggesting that the transduction efficiencies of the Ad35 vectors at these doses would not be saturated. Apparent toxicities were not observed in the cells after transduction at these doses (data not shown). These results indicate that penton base RGD motifs are largely involved with Ad35 vector-mediated transduction in both cells.

Table 1 Mutation in the penton base of Ad35 vectors

Ad35 vectors	*Amino-acid sequence of penton base*
Ad35GFP (conventional Ad35)	—NAGEV**RGD**NFAPT—
Ad35RGE-GFP (an amino acid substitution)	—NAGEV**RGE**NFAPT—
Ad35ΔRGD-GFP (deletion of RGD motif)	—NAGEV - - - - - - - - - NFAPT—

Abbreviations: Ad, adenovirus; GFP, green fluorescence protein; RGD, Arg-Gly-Asp.

Next, we compared the cellular binding and uptake of Ad35GFP, Ad35RGE-GFP and Ad35ΔRGD-GFP in the CD34+ cells and K562 cells by real-time PCR analysis to examine why the mutation of penton base RGD motifs decreased the transduction efficiencies. We found that 3 h (CD34+ cells) or 1.5 h (K562 cells) following infection, the Ad35 vector genome levels associated with both cells were comparable to those of Ad35GFP, Ad35RGE-GFP and Ad35ΔRGD-GFP at 4 and 37 °C (Figure 7), although the amount of Ad35ΔRGD-GFP associated with the CD34+ cells at 37 °C was significantly higher than those of the other vectors. These results indicate that penton base RGD motifs play an important role in postinternalization steps.

Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ and α_5 are involved with Ad35 vector infection

To determine which types of integrins participate in Ad35 vector infection in the CD34+ cells and K562 cells, we performed an infection-blocking experiment using several anti-integrin antibodies. Among the several anti-integrin antibodies, incubation with anti-$\alpha_v\beta_3$ antibody significantly reduced the GFP expression level by 41% in the CD34+ cells (Figure 8a). The other types of anti-integrin antibodies did not inhibit Ad35 vector-mediated

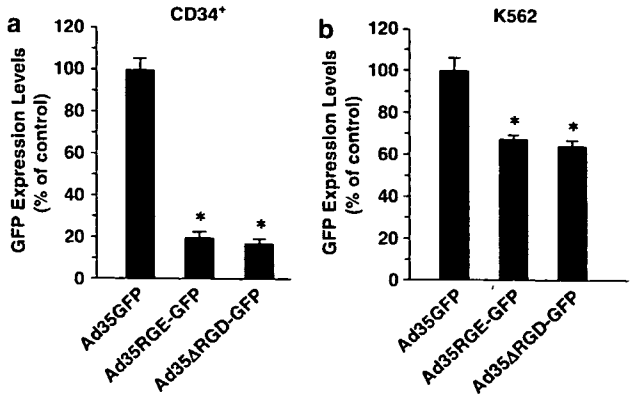

Figure 6 Effects of modification of penton RGD motifs on transduction efficiencies of Ad35 vectors. Human CD34$^+$ cells (**a**) and K562 cells (**b**) were transduced with Ad35GFP, Ad35RGE-GFP and Ad35ΔRGD-GFP at 6000 VP per cell for 6 h (human CD34$^+$ cells) or at 3000 VP per cell for 1.5 h (K562 cells) at 37 °C. GFP expression levels (MFI) were measured 48 h after transduction using flow cytometry. The GFP expression levels of Ad35GFP in the CD34$^+$ cells and K562 cells were 432.4 and 3232.7, respectively. The GFP expression levels in the mock-infected cells were 3.2 (CD34$^+$ cells) and 3.3 (K562 cells). The data were normalized to the GFP expression levels by Ad35GFP. Data are expressed as means ± s.d. ($n = 3$). *$P < 0.001$ in comparison with Ad35GFP. GFP, green fluorescence protein; MFI, mean fluorescence intensity; RGD, Arg-Gly-Asp.

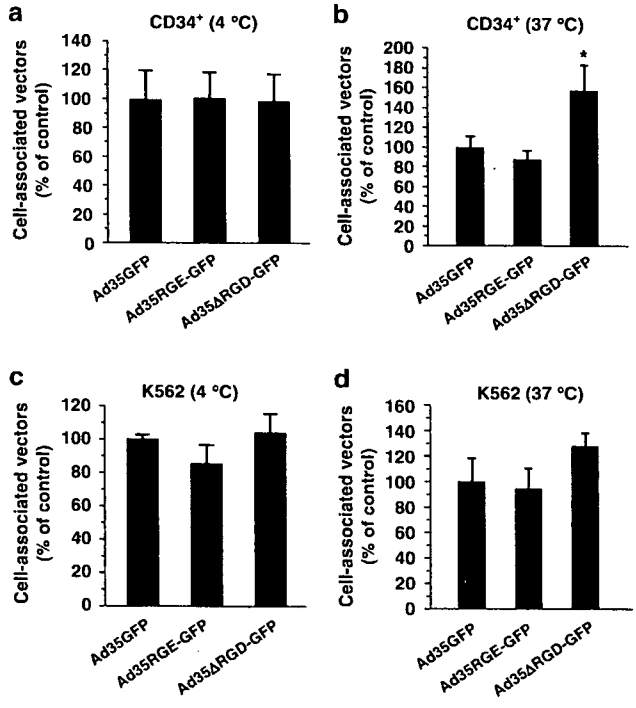

Figure 7 Effects of modification of penton RGD motifs on cellular binding and uptake of Ad35 vectors. Human CD34$^+$ cells (**a**, **b**) and K562 cells (**c**, **d**) were incubated with Ad35GFP, Ad35RGE-GFP and Ad35ΔRGD-GFP as described in Figure 6 at 4 or 37 °C. After incubation, the cells were washed five times with ice-cold phosphate-buffered saline, and total DNA, including the Ad vector DNA, was extracted. The vector copy numbers were quantified by real-time PCR. The data were normalized to the vector copy number of Ad35GFP. Data are expressed as means ± s.d. ($n = 4$). *$P < 0.05$ in comparison with Ad35GFP. GFP, green fluorescence protein; RGD, Arg-Gly-Asp.

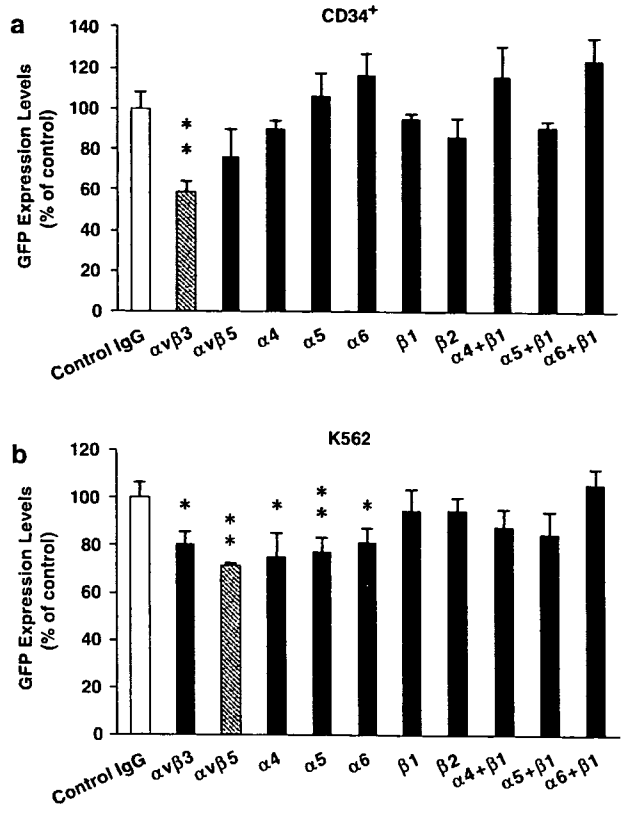

Figure 8 Inhibition of Ad35 vector-mediated transduction by monoclonal anti-integrin antibodies. Human CD34$^+$ cells (**a**) and K562 cells (**b**) were transduced with Ad35GFP at 3000 VP per cell for 3 h (human CD34$^+$ cells) or at 300 VP per cell for 0.5 h (K562 cells). GFP expression levels were measured 48 h after transduction using flow cytometry. The GFP expression levels (MFI) in the presence of control IgG were 427.0 (CD34$^+$ cells) and 24.9 (K562 cells). The GFP expression levels in the mock-infected cells were 2.8 (CD34$^+$ cells) and 2.7 (K562 cells). The data were normalized to the GFP expression levels (MFI) in human CD34$^+$ cells and K562 cells in the presence of control mouse IgG. Data are expressed as means ± s.d. ($n = 3$). *$P < 0.05$, **$P < 0.01$ in comparison with control IgG. GFP, green fluorescence protein; IgG, immunoglobulin G; MFI, mean fluorescence intensity

transduction in the CD34$^+$ cells. In K562 cells, Ad35GFP showed 20, 30 and 22% reduced levels of GFP expression in the presence of anti-$\alpha_v\beta_3$, -$\alpha_v\beta_5$ and -α_5 antibodies, respectively (Figure 8b). Pretreatment with anti-α_4 and anti-α_6 antibodies in K562 cells also significantly decreased GFP expression by Ad35GFP, in spite of undetectable expression levels of these integrins in K562 cells (see Figure 1). These results indicate that the integrins described above are involved with Ad35 vector-mediated transduction in the CD34$^+$ cells or K562 cells.

Discussion

The goal of this study was to evaluate the involvement of interaction between penton base RGD motifs and integrins on the cell surface with Ad35 vector-mediated transduction in human hematopoietic cells. To this end, we performed transduction with Ad35 vectors in hematopoietic cells in the presence of EDTA, divalent

cations and RGD peptide. Ad35 vectors with a mutation in the penton base were constructed, and transduction properties of the mutant Ad35 vectors were examined. Furthermore, Ad35 vectors transduced hematopoietic cells in the presence of several types of anti-integrin antibodies.

The interaction of penton base RGD motifs with α_v integrins is well known to facilitate the internalization of Ad5 vectors via clathrin-mediated endocytosis.[28] Several types of integrins, such as $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_1$, mediate efficient transduction with Ad5 vectors.[2–6] The structures of the Ad5 fiber and penton base allow easy access of penton base RGD motifs to integrins on the cell surface. Ad5 possess a long (22 β-repeats), and flexible fiber shaft.[29,30] The RGD-containing hypervariable loop of the Ad5 penton base is longer than those of other serotypes,[20,21] and protrudes above the penton base.[31] In contrast, the fiber shaft of Ad35 is short (7 β-repeats).[30] The hypervariable loop of the Ad35 penton base is 19 amino-acid shorter than that of Ad5. These properties of Ad35 suggest that the RGD motifs in the Ad35 penton base might be less easily associated with integrins on the cell surface. The present study indicates that the interaction of penton base RGD motifs in Ad35 with integrins is not largely involved with the cellular binding and uptake of Ad35. Segerman *et al.*[32] and Tuve *et al.*[33] also demonstrated that the presence of divalent cations, which increases the affinity of integrins to their ligands, did not affect the cellular binding of subgroup B Ads. Properties of CD46 also contribute to the lack of necessity of interaction between penton base RGD motifs and integrins for uptake of Ad35. Gaggar *et al.*[11] suggested that CD46 is not only an attachment receptor, but also a receptor for internalization of Ad35. The cytoplasmic tail of CD46 possesses a signal sequence for endocytosis.[34]

The experimental method used in this study does not allow exact evaluation of the internalized Ad35 vector copy numbers, because the amounts of the vector copy numbers at 37 °C contains both the virus particles attached on the cell surface and those truly internalized into the cells. However, it is unlikely that the amounts of Ad35 vector particles bound on the cell surface are dramatically different between 4 and 37 °C, because CD46 expression levels on the cell surface did not decrease at the Ad35 vector dose used in this study (data not shown). Therefore, we can roughly estimate that the amounts of the internalized Ad35 vector particles are not dramatically different in the presence or absence of EDTA, Mn^{2+} or the RGD peptide, assuming that the amounts of the Ad35 vector particles bound on the cell surface at both temperatures are the same.

However, interaction of penton base RGD motifs with integrins might facilitate the internalization rates of CD46-utilizing Ad vector particles into cells, especially shortly after virus infection. Deletion of penton base RGD motifs in chimeric Ad5 vectors containing the Ad35 fiber knob showed 15-min delays in the postinfection uptake of the vectors.[35] Ad serotype 41, which lacks an RGD motif on the penton base, undergoes delayed uptake in A549 cells.[21]

On the other hand, inhibition of interaction between the RGD motifs and integrins significantly decreased the transduction activity of Ad35 vectors, indicating that binding of the RGD motifs to integrins is largely involved with the transduction process of Ad35 vectors. Our group also confirmed that fiber-substituted Ad5 vectors bearing the fiber shaft and knob of Ad35 with deletion of penton base RGD motifs showed reduced transduction efficiencies compared with the parent fiber-substituted Ad5 vectors (data not shown). Shayakhmetov *et al.*[35] also demonstrated that mutation of penton base RGD motifs in chimeric Ad5 vectors containing the fiber knob of Ad35 significantly decreased the transduction efficiencies. These results indicate that penton base RGD motifs are important for transduction with not only subgroup B Ad vectors, but also CD46-utilizing chimeric Ad5 vectors. It remains unclear which process is facilitated by interaction between Ad35 penton base RGD motifs and integrins. However, the present study revealed that interaction of the RGD motifs with integrins did not significantly enhance cellular binding and uptake of Ad35, suggesting that postinternalization steps, such as escape from endosomes/lysosomes, would be accelerated by the interactions. This hypothesis is supported by the study by Shayakhmetov *et al.*,[35] which demonstrated that deletion of penton RGD motifs in chimeric Ad5 vectors possessing the fiber knob of Ad35 decreased escape from the endosomes/lysosomes. The interaction of $\alpha_v\beta_5$ integrins with penton RGD motifs was reported to enhance the escape of Ad5 from endosomes.[4]

Although significant decreases in the transduction activities were found in Ad35 vectors with a mutation of the RGD motifs, Ad5 vectors with deletion of penton base RGD motifs exhibited approximately comparable levels of transgene expression in CAR-positive cells with normal Ad5 vectors.[35,36] The difference between Ad5 and Ad35 vectors in their need for penton base RGD motifs for efficient transduction might be due to the difference in their ability to escape from endosomes. Ad5 vectors are released from endosomes immediately after endocytosis; in contrast, chimeric Ad5 vectors possessing the fiber knob of Ad35, which are supposed to show similar intracellular trafficking to Ad35 vectors, remained longer in late endosomes/lysosomes than conventional Ad5 vectors.[37]

Blocking studies using anti-integrin antibodies revealed that integrins involved with Ad35 vector infection are $\alpha_v\beta_3$ in the $CD34^+$ cells and $\alpha_v\beta_3$, $\alpha_v\beta_5$ and α_5 in K562 cells (anti-α_4 and anti-α_6 antibodies showed statistically significant inhibition in Ad35 vector-mediated transduction; however, expressions of α_4 and α_6 integrins are below detectable levels, as commented in the result section). Before the experiments, we had speculated that $\alpha_5\beta_1$ integrin was mainly involved with Ad35 vector-mediated transduction, because human bone marrow $CD34^+$ cells expressed integrins α_5 and β_1. In addition, the affinity of ligands to integrins is influenced by the amino acid following the RGD sequence while the RGDN (Arg-Gly-Asp-Asn) sequence, which is contained in the Ad35 penton base, has a high affinity for $\alpha_5\beta_1$ integrin.[38] However, the efficiencies of Ad35GFP transduction did not decrease in the presence of anti-α_5 or anti-β_1 antibodies in the $CD34^+$ cells, although preincubation with anti-α_5 antibody reduced transduction of Ad35GFP in K562 cells. It remains unclear why $\alpha_5\beta_1$ integrin is not involved with Ad35 vector infection in the $CD34^+$ cells. In addition, only the anti-$\alpha_v\beta_3$ antibody inhibited transduction with Ad35 vectors in the $CD34^+$ cells, and anti-integrin antibodies used in this study showed low levels of inhibition in both cells. Other types

of integrins which were not tested in this study, or unknown receptor(s), might be involved with Ad35 infection.

In summary, we demonstrated that the interaction between penton base RGD motifs and integrins are important for Ad35 vector-mediated transduction in hematopoietic cells. The results of our study contribute to a better understanding of the mechanism of Ad35 vector-mediated transduction and may offer valuable information for the development of more efficient Ad vectors.

Materials and methods

Cells and antibodies

Human bone marrow-derived $CD34^+$ cells (Cambrex Bio Science Inc., Walkersville, MD, USA) were recovered from the frozen stock 16–18 h before transduction, and suspended in StemSpan 2000 containing the cytokine cocktail StemSpan CC100 (human Flt-3 ligand (100 ng ml^{-1}), human stem cell factor (100 ng ml^{-1}), human interleukin-3 (20 ng ml^{-1}) and human interleukin-6 (20 ng ml^{-1})) (StemCell Technologies Inc., Vancouver, BC, Canada). K562 cells (human chronic myelogeneous leukemia from blast crisis) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Purified function-blocking monoclonal antibodies to integrins $\alpha_v\beta_3$ (LM609), $\alpha_v\beta_5$ (P1F6), α_5 (P1D6), β_1 (P4C10) and β_2 (P4H9-A11) were purchased from Chemicon International (Temecula, CA, USA). Monoclonal antibodies to integrin α_4 (HP2/1) and α_6 (GoH3) were obtained from Immunotech (Marseille, France) and R&D Systems Europe (Abingdon, UK), respectively.

Plasmids

Ad35 vector plasmid pAdMS18 was constructed as follows. The *Sbf*I/*Asc*I fragment of pAdMS1,[15] which is the left end of the Ad35 genome (1–7930 bp), was ligated with the *Sbf*I/*Asc*I fragment of pFS2,[15] resulting in pFS2-Ad35-12. pFS2-Ad35-12 was cut by *Sna*BI and *Pac*I, and ligated with oligonucleotides 1 (5′-TAACTATAACGGTC CTAAGGTAGCGA*ATTTAAAT***ATCTATGTCGGGTGCG GAGAAAGAGGTAATGAAATGGCA**AT-3′) and 2 (5′-T **GCCATTTCATTACCTCTTTCTCCGCACCCGACATAG AT***ATTTAAAT***T**CGCTACCTTAGGACCGTTATAGTTA-3′) (I-*Ceu*I, *Swa*I and PI-*Sce*I recognition sequences are noted by underlining, italics and bold, respectively), resulting in pFS2-Ad35-13, which contains I-*Ceu*I, *Swa*I and PI-*Sce*I sites in the E1 deletion site of the Ad35 genome. The I-*Ceu*I/*Asc*I fragments of pFS2-Ad35-13 and pAdMS4[23] were ligated, resulting in pAdMS18. pAdMS18 has I-*Ceu*I, *Swa*I and PI-*Sce*I sites in the E1 deletion region (ΔE1:450–2912 bp).

Vector plasmids pAdMS19 and pAdMS20, which were used to generate Ad35 vectors containing an amino-acid substitution in the penton RGD sequence (D343E) or a deletion of the RGD motif in the penton base, respectively, were constructed using the following procedures. pFS5-Ad35-2 was constructed by ligation of the *Asc*I/*Eco*RI fragment (7930–21 944 bp) of the Ad35 genome and the *Asc*I/*Eco*RI fragment of pFS5. pFS5 was constructed by ligation of *Xba*I/*Sac*I-digested pHM5[39] with the oligonucleotides containing the multicloning sites. A shuttle plasmid pFS4, which contains the multicloning site, *Pme*I/*Asc*I/*Nhe*I/*Bst*1107I/*Csp*45I/*Pac*I/*Not*I, was digested with *Sph*I/*Csp*45I and ligated with the *Sph*I/*Csp*45I fragment of pFS5-Ad35-2, resulting in pFS4-Ad35-1. pFS4 was constructed by ligation of *Sph*I/*Sal*I-digested pHM5 with oligonucleotides containing the multicloning site. pFS4-Ad35-2, which has the Ad35 genome (14 409–15 544 bp), was constructed by ligation of *Bst*1107I/*Csp*45I fragments of pFS4-Ad35-1 and pFS4. Then, pFS4-Ad35-2 was cut by *Pvu*II and *Pst*I, and ligated with oligonucleotides 3 (5′-CTGCTGCA GAAGCTAAGGCAAACATAGTTGCCAGCGACTCTAC AAGGGTTGCTAACGCTGGAGAGGTCAGAGGAGAG AATTTTGCGCCAACACCTGTTCCGACTGCA-3′) and 4 (5′-GTCGGAACAGGTGTTGGCGCAAAATTCTCTCCTC TGACCTCTCCAGCGTTAGCAACCCTTGTAGAGTCGC TGGCAACTATGTTTGCCTTAGCTTCTGCAGCAG-3′) (the RGE sequence is underlined), resulting in pFS4-Ad35-5. pFS4-Ad35-5 was digested with *Sph*I/*Pvu*II and ligated with the *Sph*I/*Pvu*II fragment of pFS4-Ad35-1, resulting in pFS4-Ad35-6. pFS4-Ad35-7, which was constructed by ligation of the I-*Ceu*I/*Blp*I fragment of pFS5-Ad35-2 and I-*Ceu*I/*Blp*I-digested pFS4-Ad35-6, were cut by *Sgr*AI/*Pac*I and ligated with the *Sgr*AI/*Pac*I fragment of pFS5-Ad35-2, resulting in pFS4-Ad35-9. Finally, pAdMS19 was constructed by homologous recombination of the *Asc*I/*Pac*I fragment of pFS4-Ad35-9 with *Bst*1107I-digested pAdMS18 in *Escherichia coli* BJ5183. pAdMS20 was similarly constructed using oligonucleotides 5 (5′-CTGCTGCAGAAGCTAAGGCA AACATAGTTGCCAGCGACTCTACAAGGGTTGCTAA CGCTGGAGAGGTCAATTTTGCGCCAACACCTGTTCC GACTGCA-3′) and 6 (5′-GTCGGAACAGGTGTTGGCG CAAAATTGACCTCTCCAGCGTTAGCAACCCTTGTA GAGTCGCTGGCAACTATGTTTGCCTTAGCTTCTGCA GCAG-3′).

Virus

The Ad35 vectors were prepared by means of an improved *in vitro* ligation method described previously.[23,39,40] GFP-expressing Ad35 vector plasmids (pAdMS18CA-GFP, pAdMS19CA-GFP and pAdMS20CA-GFP) were constructed by ligation of I-*Ceu*I/PI-*Sce*I-digested pAdMS18, pAdMS19 and pAdMS20, respectively, and I-*Ceu*I/PI-*Sce*I-digested pHMCA5-GFP. pHMCA5-GFP was constructed by insertion of the GFP gene, which was derived from pEGFP-N1 (Clontech, Mountain View, CA, USA) into pHMCA5.[41]

To generate the virus, pAdMS18CA-GFP, pAdMS19-CA-GFP and pAdMS20CA-GFP were digested with *Sbf*I and purified by phenol–chloroform extraction and ethanol precipitation. Linearized plasmids were transfected into 293E1B cells[42] with SuperFect (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Determination of virus particle titers was accomplished spectrophotometrically by the method of Maizel *et al*.[43]

Flow cytometric analysis of integrin expressions

Human $CD34^+$ cells and K562 cells suspended in staining buffer (phosphate-buffered saline buffer containing 1% bovine serum albumin) were incubated with mouse anti-human integrin $\alpha_v\beta_3$, $\alpha_v\beta_5$, α_4, α_5, α_6, β_1 and β_2 antibodies for 1 h. Subsequently, the cells were reacted with phycoerythrin-labeled secondary anti-mouse IgG antibody (Pharmingen, San Diego, CA, USA). After washing

with the staining buffer, the stained cells (10^4 cells) were analyzed using FACSCalibur and CellQuest software (Becton Dickinson, Tokyo, Japan).

Transduction experiments

Human $CD34^+$ cells and K562 cells were seeded at 1×10^4 cells per well into a 96-well plate. The cells were transduced with Ad35GFP, Ad35RGE-GFP or Ad35ΔRGD-GFP at 6000 VP per cell for 6 h ($CD34^+$ cells) or 3000 VP per cell for 1.5 h (K562 cells). After a 48 h culture period, GFP expression levels were measured using flow cytometry as described above.

To examine the effects of divalent cations on Ad35 vector-mediated transduction, K562 cells were preincubated in Tris-buffered saline buffer containing $MnCl_2$, $MgCl_2$ or $CaCl_2$ at indicated concentrations for 30 min at 4 °C prior to transduction. Subsequently, the cells were transduced with Ad35GFP at 1000 VP per cell for 1.5 h at 37 °C. After a 1.5 h incubation, the cells were washed and resuspended in a fresh medium. GFP expression levels were measured using flow cytometry following a total of 48 h of the incubation as described above. In blocking experiments using the RGD peptide, human $CD34^+$ cells and K562 cells were preincubated in the medium containing synthetic RGD peptide (GRGDSP, GRGESP; TaKaRa, Osaka, Japan) at 4 °C for 1 h. Subsequently, the cells were transduced with Ad35GFP at 3000 VP per cell for 3 h ($CD34^+$ cells) or 1000 VP per cell for 1 h (K562 cells) at 37 °C after which the cells were washed, resuspended in a fresh medium and incubated at 37 °C GFP expression levels were measured 48 h after transduction as described above. Blocking experiments were similarly performed using monoclonal anti-human integrin antibodies. Following incubation with anti-human integrin antibodies (50 μg ml^{-1}), the cells were transduced with Ad35GFP at 3000 VP per cell for 3 h ($CD34^+$ cells) or 300 VP per cell for 0.5 h (K562 cells). GFP expression levels were evaluated as described above.

Cellular binding and uptake of Ad35 vectors

For evaluation of effects of divalent cations on cellular binding and uptake of Ad35 vectors, K562 cells, which were seeded at 3×10^4 cells well $^{-1}$ into a 48-well plate, were incubated with Ad35GFP in the presence of 5 mM EDTA or Mn^{2+} for 1.5 h at 4 or 37 °C as described above. The cells were then recovered and washed five times with ice-cold phosphate-buffered saline, and total DNA, including the Ad vector DNA, was extracted from the cells using DNeasy Tissue Kit (Qiagen). Quantitative real-time PCR was performed as described previously.[23] In transduction-blocking experiments using the RGD peptide, human $CD34^+$ cells and K562 cells were incubated with Ad35GFP at 4 or 37 °C in the presence of 200 μg ml^{-1} of the RGD peptide for 3 h ($CD34^+$ cells) or 1.5 h (K562 cells) as described above. Following incubation, the cells were washed and the vector genome numbers were measured by real-time PCR analysis, as described above. The amounts of vector genome of Ad35GFP, Ad35RGE-GFP and Ad35ΔRGD-GFP were similarly evaluated following incubation with Ad35GFP, Ad35RGE-GFP and Ad35ΔRGD-GFP at 6000 VP per cell for 3 h ($CD34^+$ cells) or 3000 VP per cell for 1.5 h (K562 cells) at 4 or 37 °C.

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＊総　　説＊

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癌に対する抗血管新生療法の現状と展望（その1）

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State and Perspective of Anti-Angiogenic Therapy to Cancer 1

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はじめに

血管新生は固形腫瘍の成長と進展において必須のステップであり，癌の主な死亡原因である．約30年前，Folkmanは腫瘍の増殖は血管新生のプロセスに依存していることを示した[1]．腫瘍が数ミリ以上の直径に達して進行するには血液を介した酸素及び栄養物の供給が必要であるが，両者の距離が離れているとその供給は困難である．そこで，腫瘍の成長及び進行には新しい血管の供給が必要であり，新しい血管新生のブロックにより腫瘍の成長を抑制できることを彼は提唱した．現在この概念は多くの研究により支持されている．その数年後，Gullinoは前癌状態の組織における細胞は血管新生能を獲得して癌になることを示し，血管新生の阻害は癌の抑制に用いることができることを提唱した[2]．また，血管新生阻害療法が癌の治療法として有用であると考えられている理由は以下の点である．1個の腫瘍血管内皮細胞により約100個の腫瘍細胞が養われている．したがって，1個の腫瘍内皮細胞を死なせることにより，100個の腫瘍細胞を死なすことができる．腫瘍血管内皮細胞は正常細胞なので薬剤耐性を獲得しないため，治療を中止後再発した場合でも最初と同じ血管新生阻害剤が有効なはずである．腫瘍細胞は多様であるため多種類の抗癌剤が必要であるが，血管内皮細胞は基本的に同じ性質をもつ正常細胞であるので，1種類の有効な阻害剤があらゆる腫瘍に有効である可能性がある．

過去30年で，一般的な血管新生，特に腫瘍の血管新生に対する我々の理解は急激に広がっており，ついに進行性の結腸直腸癌の治療に対し最初の抗血管新生治療薬としてBevacizumabが承認された．また，様々な抗血管新生治療薬を用いた60以上もの臨床試験が行われている[3~7]．

広く受け入れられている抗血管新生の作用機構は，これら薬剤が新しい血管の形成を阻害することにより腫瘍の成長と転移を抑制するというものである．残念ながら，最近の無作為臨床試験からの結果によると，利用が可能な抗血管新生治療薬を単独で用いても中程度の客観的な奏功しか示せず，生存率を長期間にわたり延長できなかった[8~10]．しかし，化学療法剤との併用により，VEGFに対する抗体であるBevacizumabは転移性の結腸直腸癌の患者において，生存期間を5箇月も増加させた[11]．

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このように血管新生治療薬と化学療法剤との併用が癌の治療法として有用であるのは，抗血管新生治療薬が腫瘍血管を正常な血管に改善し，酸素及び薬の腫瘍細胞に対するデリバリーを改善することによるという仮説が提唱されている．本稿ではこの新しい仮説に基づく抗血管新生治療として，たん白質及びペプチド単独あるいは化学療法剤との併用を用いた非臨床及び臨床研究の現状及び展望そして腫瘍における血管新生に関する知見について概説する．なお，本稿は成書を参考にした[3,4,7,12~17]．

1．腫瘍の進行における血管新生の役割

成人において，正常に起きる新血管形成は女性の生殖器官及び創傷治癒に主に限定される．そうでない場合，成人における新血管形成は癌のような病理的な状況になる．最初の段階における腫瘍の成長には正常血管の取り込みが必要であるが，腫瘍血管が発達する以前では腫瘍は休眠している[18]．血管新生が起きるには，血管新生促進因子の効果が抗血管新生因子の効果を上回らなければならない．そのバランスが血管新生促進に傾くと，新しい血管の発達が促進され，腫瘍において血管新生を進行させるスイッチが入る[19]．

いったん血管が発達すると，腫瘍は転移の表現系に向かって急速に増殖し進行する．最近のデータによると，腫瘍，循環内皮細胞，内皮前駆細胞及び腫瘍細胞[20,21]は増殖因子，サイトカイン，ホルモン[22]及び浸潤促進活性を有する因子[23,24]を遊離し，それらの因子により腫瘍の成長及び転移が促進されることが示唆されている．更に，腫瘍の血管系においては，これら細胞が新生血管を介して血管内に入り，その後離れた毛細血管から定着した転移性病巣に移動する際，転移性癌細胞播種のルートを提供する[25~27]．癌の進行における血管新生の役割は，腫瘍内の微小血管密度及び一定の領域における血管の数が，ある種の腫瘍型において予後因子となるだけでなく転移の進展とも相関することを示す研究から明らかになっている[28]．

2．腫瘍における血管新生の機構

腫瘍の血管形成は，少なくても吸収，陥入，出芽（血管新生），脈管形成という4種類の機構により起こる．腫瘍細胞は既存の血管を吸収してその周りで増殖し，血管周囲にカフを形成する．しかし，重要な栄養物が限界以上に拡散するとカフは成長できず，腫瘍細胞の成長により生じる圧縮力により血管が崩壊する．あるいは，腫瘍により放出される増殖因子による既存の血管の肥大，管腔の分割といった一連のプロセスにより拡大した血管ネットワークが形成される．このような，陥入した微小血管の成長は腫瘍の成長，創傷治癒でも観察される[29~32]．

血管形成のなかで最も広く研究されている分野は，血管新生の機構であると考えられる．血管新生の機構及び血管新生促進因子の詳細については成書[33]を参考にされたい．以下に血管新生の機構の要約を示す．血管新生の間，既存の血管は，正常細胞あるいは癌細胞から遊離される増殖因子に反応して透過性が亢進し，基底膜及び間質マトリックスの分解，周皮細胞の血管からの解離，内皮細胞の遊走及び増殖により血管の配列あるいは出芽が形成され，管腔は出芽の中で形成される．出芽の集合及び吻合により枝及びループが形成され，血流が形成される．これら未熟な血管は基底膜及び周皮細胞に取り込まれ，このプロセスはカナリゼーションと呼ばれる．正常な生理学的な血管新生の間では，これら血管は成熟細動脈，毛細血管，小静脈に分化する．一方，これら血管は腫瘍において未熟のままである[34~37]．最終的に，内皮前駆細胞から形成された血島が互いに連絡しあい，胎児の発育中に原始的な血管網が形成される．骨髄あるいは末梢血から動員される循環内皮前駆細胞は腫瘍及び他の組織における生後の脈管形成にも寄与する[38~40]．腫瘍の治療における最近の課題は，腫瘍の形成に対する血管新生促進の四つの機構それぞれについて相対的な役割を明確に理解し，癌の抗血管新生治療を最適化することである[41]．

3．腫瘍血管の構造と機能

腫瘍の成長と転移において血管は重要な役割を果たしている．そして腫瘍血管系の構造及び機能は異常である．その内皮内面は不完全であり，開窓及び細胞間接合は喪失している．基底膜は不連続なものとして存在するかあるいは消失し，周囲の周皮細胞は欠如している．組織化された血管ネットワークの構造は消失している．血管ネットワークにおいてははっきりとした細動脈，小静脈，毛細血管が欠如しており，血管の間のつながりが不完全な場合がある．