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The short consensus repeats 1 and 2, not the cytoplasmic domain, of human CD46 are crucial for infection of subgroup B adenovirus serotype 35

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Abstract

Human CD46 (membrane cofactor protein) has recently been identified to be an attachment receptor for subgroup B adenoviruses (Ads); however, the precise interaction between human CD46 and subgroup B Ads are just beginning to be understood. In this study, to characterize the interaction between human CD46 and subgroup B Ads, varieties of mutant CD46 were tested for their ability to act as a receptor for Ad serotype 35 (Ad35), which belongs to subgroup B. In addition, we determined Ad35 vector-mediated transgene expression and cellular uptake of Ad35 vectors in the presence of a set of anti-CD46 antibodies. Our data demonstrated that the short consensus repeats (SCRs) 1 and 2 in human CD46 are important for interaction with Ad35, whereas the cytoplasmic domain of human CD46 was found not to be required for the function as an Ad35 receptor. Rather, a complete deletion of the cytoplasmic domain of human CD46 increased the transduction efficiencies of Ad35 vectors. This information should help in elucidation of the mechanism of subgroup B Ad infection, as well in the improvement of the subgroup B Ad vectors.

Keywords: Adenovirus serotype 35 vector; CD46; Short consensus repeat; Cytoplasmic tail; Gene therapy

1. Introduction

Human adenoviruses (Ads) compose a large family of non-enveloped, double-stranded DNA viruses that are a significant cause of acute respiratory, gastrointestinal, and ocular infections in humans. So far, at least 51 serotype Ads have been identified and classified into six distinct subgroups (A–F) [1,2]. Among them, subgroup B is further subdivided into subspecies B1 and B2 on the basis of various biophysical and biochemical criteria. Among the 51 human Ad serotypes, the Ad vector most commonly used for gene transfer is composed of Ad serotype 5 (Ad5), which belongs to subgroup C. Ad5 vectors are very powerful and useful vehicles, but recent studies have revealed that they also have some disadvantages, such as high seroprevalence toward Ad5 in adult populations and low infection activity in cells lacking a primary receptor for Ad5, coxsackievirus and adenovirus receptor (CAR). On the other hand, subgroup B Ads have unique properties that are distinct from those of other subgroup Ads, and that are highly attractive features as a framework for alternative gene delivery vehicles. First, subgroup B Ads have been identified as having lower prevalence than the Ads of other subgroups. The seroprevalences toward most subgroup B Ads is less than 20% in healthy blood donors, while more than 70% of serum samples from healthy donors are positive for anti-Ad5 antibody [3]. This indicates that transduction with Ad vectors based on subgroup B is unlikely to be inhibited by preexisting anti-Ad antibodies. Second, subgroup B Ads utilize human CD46 (membrane cofactor protein) as a cellular receptor for infection [4,5], while other subgroup Ads recognize CAR. Human CD46 is ubiquitously expressed in human cells, suggesting that subgroup

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B Ad vectors would have a broad tropism for human cells. We have previously developed an Ad vector composed of Ad serotype 35 (Ad35), which belongs to subgroup B [6,7], and have demonstrated that Ad35 vectors exhibit a wider tropism for human cells, including CAR-negative cells, than Ad5 vectors [7].

Human CD46 is a type I transmembrane glycoprotein expressed in almost all human cells, except for erythrocytes. Human CD46 is composed of four cysteine-rich short consensus repeats (SCRs), a serine–threonine–proline-rich (STP) region, a short region of unknown function, a hydrophobic transmembrane domain, and a carboxy-terminal cytoplasmic domain. Alternative splicing in the STP region and the cytoplasmic domain gives rise to four major isoforms of human CD46 (BC1, BC2, C1, and C2). All the isoforms function as cofactors for the plasma serine protease factor I by binding to the complement factors C3b and C4b deposited on self tissue [8,9]. By promoting the proteolytic degradation of these factors, these isoforms protect the cells from complement attack [10,11]. In addition to this function, human CD46 has been identified to be a receptor for several human pathogens: measles virus (MV), human herpesvirus 6 (HHV6), human subgroup B Ads, and two types of bacteria [4,5,12–15]. Among these pathogens, the interactions between human CD46 and MV, HHV6, and pathogenic *Neisseria* have been well studied. MV-binding residues are located on SCR1 and SCR2 [16,17], while SCR3 and 4 are essential for binding of HHV6 to human CD46 [18]. The cytoplasmic domain of CD46 is not required for infection of both MV and HHV6 [18,19]. However, it still remains unknown which domains in human CD46 play an important role in the interaction with subgroup B Ads. Elucidation of the interaction between subgroup B Ads and CD46 would lead to improvement of the Ad vectors that are composed of subgroup B Ads.

In this study, the transduction experiments with Ad35 vectors expressing luciferase were performed using cells expressing a variety of human CD46 mutants in order to map the domains which interact with Ad35. Furthermore, cells expressing wild-type CD46 were transduced with Ad35 vectors in the presence of monoclonal anti-human CD46 antibodies which recognize different SCRs of human CD46. Finally, involvement of the cytoplasmic domain of human CD46 with infection of Ad35 was evaluated.

2. Materials and methods

2.1. Cells and antibodies

Chinese hamster ovary (CHO) cells and CHO transformants stably expressing wild-type CD46, CD46 SCR deletion mutants [16], or cytoplasmic tail deletion mutants were grown in Ham's F-12 medium with 10% fetal bovine serum. Cytoplasmic tail deletion mutants (ΔCyt0 and ΔCyt6 mutants) were stable CHO transformants generated by the transfection of pcDNA-CD46ΔCyt0 and pcDNA-CD46ΔCyt6 (described below) into CHO cells and selection with hygromycin (GIBCO-BRE, Rockville, MD). Monoclonal antibodies against human CD46 SCR1, E4.3, MEM-258, and J4-48 were purchased from Pharmingen (San Diego, CA), Serotec Ltd. (Oxford, United Kingdom), and Immunotech (Marseille, France), respectively. SCR2-specific antibody M177 and SCR3-specific antibody M160 were described previously [20]. The monoclonal anti-CD46 antibodies used in this study and their recognition sites are listed on Table 1.

2.2. Plasmids

The plasmid pcDNA-CD46C2, which contains the human CD46 C2 isoform gene, was constructed as follows. The cDNA of the human CD46 C2 isoform was amplified by PCR using the following primers: CD46-forward, 5′-ATG GAG CCT CCC GGC CGC CGC GAG TGT CCC-3′; CD46-reverse, 5′-CGC GGC CGC CTA TTC AGC CTC TCT GCT CTG CTG-3′. The PCR product was cloned into the *Pme*I site of pcDNA3.1-Hyg(+) (Invitrogen, Carlsbad, CA). The cDNA of the CD46 mutant lacking the cytoplasmic tail (amino acid residues 347–369) (CD46ΔCyt0) was prepared by PCR using the parent CD46 C2 cDNA as a template. The following primers were used for PCR: CD46-forward (described above); and CD46TM-reverse, 5′-GCG GCC GCT CAG TAC GGG ACA ACA CAA ATT ACT GCA AC-3′. The PCR product was cloned into the *Pme*I site of pcDNA3.1-Hyg(+), resulting in pcDNA-CD46ΔCyt0. The plasmid pcDNA-CD46ΔCyt6, which contains a human CD46 C2 isoform lacking a portion of the cytoplasmic domain (amino acid residues 352–369) (CD46ΔCyt6), was constructed in a similar manner using the following primers: CD46-forward (described above); and CD46TM6-reverse, 5′-GCG GCC GCT CAC CTC CTT TGA AGA TAT CTG TAC GGG AC-3′. The sequences of all the constructs were confirmed by DNA sequencing.

2.3. Flowcytometric analysis of CD46 expression

Several CHO cell transformants suspended in staining buffer (phosphate buffered saline (PBS) buffer containing 1% bovine serum albumin (BSA)) were incubated with mouse anti-human CD46 antibodies (E4.3, M177, and M160) for 1 h. Subsequently, the cells were reacted with phycoerythrin (PE)-labeled secondary anti-mouse IgG antibody (Pharmingen). After washing with the staining buffer, the stained cells (10^4 cells) were analyzed using a FACSCalibur and CellQuest software (Becton Dickinson, Tokyo, Japan). For evaluation of Ad35 vector-mediated downregulation of CD46, the CHO transformants were transduced with Ad35L at 3000 vector particles (VP)/cell for 1.5 h as described below. After a 1.5-h incubation, CD46 expression levels in the cells were measured using flowcytometry as described above.

Table 1
Monoclonal anti-CD46 antibodies used in this study

Anti-CD46 antibodies	Recognition domain
E4.3	SCR1
J4-48	SCR1
MEM-258	SCR1
M177	SCR2
M160	SCR3

2.4. Adenovirus vectors

Ad35 vectors expressing luciferase, Ad35L, were prepared by an improved ligation method as previously described [21]. Briefly, the luciferase-expressing Ad35 vector plasmid pAdMS4-CMVL2 was constructed by ligating I-*Ceu*I/PI-*Sce*I-digested pAdMS4 with I-*Ceu*I/PI-*Sce*I-digested pCMVL1 [22]. pAdMS4-CMVL2 was digested with *Sbf*I and the linearized DNA was transfected into VK10-9 cells (kindly provided by Dr. V. Krougliak) [23]. Ad35L were generated 10–14 days after transfection, amplified and purified as described previously [6,7]. Determination of virus particle titers was accomplished spectrophotometrically by the method of Maizel et al. [24].

2.5. Transduction experiments

CHO cells and CHO transformants stably expressing wild-type CD46 or CD46 mutants lacking SCRs or the cytoplasmic tail were seeded at 1×10^4 cells/well into a 96-well plate. On the following day, the cells were transduced with Ad35L at 3000 VP/cell for 1.5 h. Forty-eight hours later, luciferase productions in the cells were measured using a luciferase assay system (PicaGene LT2.0, Toyo Inki Co. Ltd., Tokyo, Japan).

For antibody blocking experiments, CHO transformant expressing CD46 C2 isoform, which was seeded at 1×10^4 cells/well in a 96-well plate the day before transduction, was preincubated with the medium containing anti-CD46 antibodies (E4.3, MEM-258, J4-48, M177, and M160) at the indicated concentrations at 4 °C for 1 h. Ad35L was then added at 3000 VP/cell and left for 1.5 h at 4 °C, after which the cells were washed and incubated at 37 °C. Luciferase productions in the cells were measured 48 h after transduction as described above.

2.6. Real-time quantitative PCR

CHO cells and CHO transformants were seeded at 1×10^5 cells/well into a 12-well plate. On the following day, the cells were transduced with Ad35L as described above. After a 48-h incubation, the cells were washed with PBS, harvested, and pelleted. Total DNA, including the Ad35 vector DNA, was extracted from the cells using a Tissue DNeasy Kit (Qiagen, Valencia, CA, USA). The quantitative real-time PCR was performed with 25 ng of sample DNA, 0.5 μM each primer, 0.16 μM TaqMan probe, and 25 μl of TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA) in a final volume of 50 μl using the ABI Prism 7000 sequence detection system (Applied Biosystems). The PCR was initially denatured at 95 °C for 10 min and then subjected to cycles of 95 °C for 15 s and 60 °C for 1 min. The reaction was carried out for 50 cycles. Primers for amplification were located in the pIX region of Ad35 genome. The sequences of the primers and probe used were as follows: forward, 5′-TGGATGGAAGACCC GTTCAA-3′; reverse, 5′-CGTCCAAAGGTGAAGAACTTA AAGT-3′; probe, 5′ FAM-CGCCAATTCTTCAACGCTGACC TATGC-TAMRA 3′. These sequences were designed using Primer Express software version 1.0 (Applied Biosystems), and it was confirmed that they amplified the products of desired size. The Ad35 vector plasmid pAdMS4 was used as a standard.

3. Results

3.1. Ad35 vector-mediated transduction on CHO transformants expressing CD46 deletion mutants

First, we examined which SCR domains of human CD46 (Fig. 1) are essential for infection of Ad35 using CHO transformants expressing CD46 deletion mutants [16]. Before the transduction experiments, CD46 expression levels and SCR deletion on CHO transformants were confirmed by flowcytometric analysis using anti-CD46 antibodies against each of the SCRs. We found the sufficient levels of CD46 mutant expression for all the clones (Fig. 2). The combined use of several anti-CD46 antibodies demonstrated that the corresponding SCR domains were properly deleted on the CHO transformants. Deletion of SCR4 on the ΔSCR4 mutant was confirmed by RT-PCR and DNA sequence, because the SCR4-specific antibody was not obtained (data not shown).

Next, transduction experiments with Ad35 vectors on CHO transformants were performed. Transduction with Ad35L in ΔSCR1 and ΔSCR2 mutants resulted in approximately 50% of the luciferase production obtained in CHO-CD46 cells, which express full-length CD46. The decreases in the transduction efficiencies in ΔSCR1 and ΔSCR2 mutants were similar. In contrast, the ΔSCR3 and ΔSCR4 mutants produced amounts of luciferase similar to those in CHO-CD46 cells after Ad35L transduction (Fig. 3). Real-time PCR analysis also demonstrated that the uptake of Ad35L was significantly reduced by 58% and by 45% in ΔSCR1 and ΔSCR2 mutants, respectively, compared with CHO-CD46 cells, in contrast, ΔSCR3 and ΔSCR4 mutants exhibited the levels of Ad35 vector uptake similar to CHO-CD46 cells (Fig. 4). These results suggested that SCR1 and 2 are involved with Ad35 infection.

3.2. Blocking of Ad35 vector infection by anti-CD46 antibodies

Next, to further examine which SCR domains in CD46 are used for Ad35 infection, several monoclonal antibodies recognizing

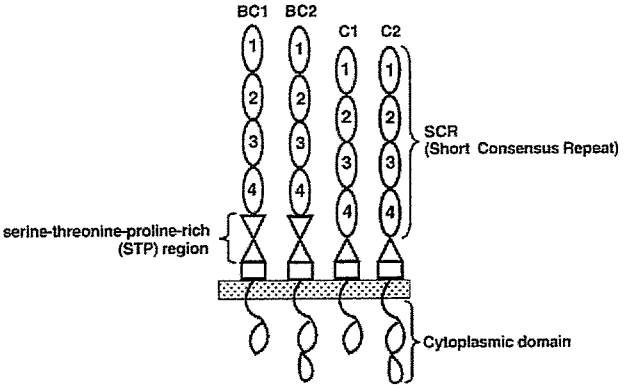

Fig. 1. A schematic diagram of human CD46. Human CD46 is ubiquitously expressed in almost all human cells mainly as four isoforms (BC1, BC2, C1, C2) that are derived via alternative splicing. Human CD46 is composed of four cysteine-rich short consensus repeats (SCRs), a serine–threonine–proline-rich (STP) region, a short region of unknown function, a hydrophobic transmembrane domain, and a carboxy-terminal cytoplasmic domain.

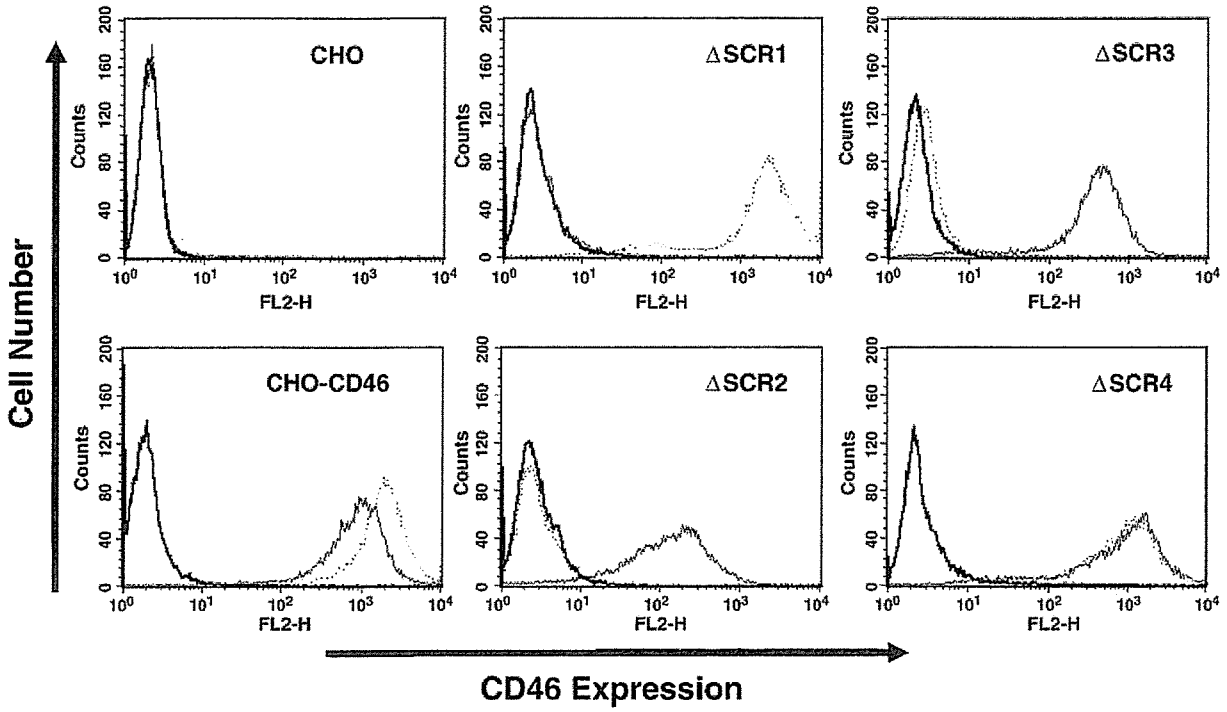

Fig. 2. Expression profiles of CD46 deletion mutants detected by monoclonal anti-CD46 antibodies. The cells were stained with anti-CD46 antibodies against SCR1 (E4.3; thin line), or SCR2 (M177; dotted line), followed by a PE-labeled secondary antibody, and subsequently analyzed by a flowcytometer. ΔSCR3 mutants were treated with anti-CD46 antibody against SCR3 (M160; dotted line) instead of M177. As a negative control, the cells were incubated with irrelevant control IgG, followed by a PE-labeled secondary antibody (thick line).

different domains of CD46 were used to block the transduction with Ad35 vectors. As shown in Fig. 5, the SCR1-specific antibody MEM-258 and the SCR2-specific antibody M177 efficiently inhibited the Ad35 vector-mediated transduction in CHO-CD46 cells. The manufacturer's information indicates that MEM-258 recognizes the SCR4 domain; however, our data indicates that MEM-258 binds to the SCR1 domain (data not shown). A recent study also reported that the epitope of MEM-258 is located in SCR1 [25]. We found that the luciferase production in the presence of both MEM-258 and M177 at 0.5 μg/ml was significantly reduced, compared with each of these antibodies alone (Fig. 5B). In contrast, the antibodies E4.3 and J4-48, which also recognize SCR1, did not decrease the luciferase production by Ad35L, suggesting that the region recognized by MEM-258, but not E4.3 and J4-48, would be involved with Ad35 infection. Decrease in the transduction efficiencies with Ad35L was not also found in the presence of the SCR3-specific antibody M160. The anti-CD46 antibodies which reduced the Ad35 vector-mediated transduction also inhibited the uptake of Ad35L by CHO-CD46 cells in a dose-dependent manner (Fig. 6). The SCR1-specific antibody MEM-258 and

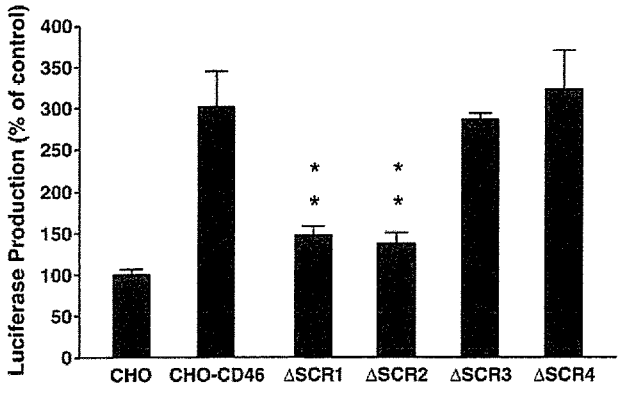

Fig. 3. Ad35L-mediated transduction in CHO cells expressing CD46 mutants lacking SCRs. The cells were transduced with Ad35L at 3000 VP/cell for 1.5 h. The luciferase productions in the cells were measured 48 h after transduction by luminescent assay. The data were normalized to the luciferase production in parental CHO cells. The absolute luciferase production in parental CHO cells was 200 pg/well. The data are expressed as the mean±S.D. (n=4). The asterisks indicate the level of significance (P<0.005 [double asterisk] for comparison with CHO-CD46 cells).

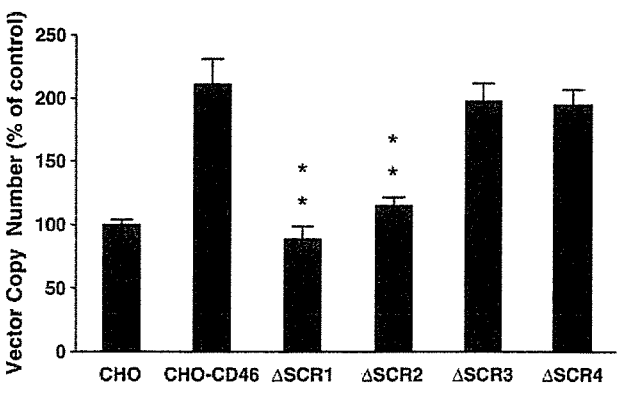

Fig. 4. Cellular uptake of Ad35L in CHO cells expressing CD46 mutants lacking SCRs. The cells were transduced with Ad35L at 3000 VP/cell for 1.5 h. The total DNA, including the vector DNA, was extracted from the cells 48 h after transduction. The copy numbers of the vector DNA were quantified by TaqMan-PCR. The data were normalized to the amounts of the vector DNA in CHO cells. The data are expressed as the mean±S.D. (n=3). The asterisks indicate the level of significance (P<0.005 [double asterisk] for comparison with CHO-CD46 cells).

the SCR2-specific antibody M177 at 5 μg/ml decreased the cellular uptake of Ad35L by 94%. These results suggest that CD46 SCR1 and SCR2 are crucial domains for Ad35 infection.

3.3. Ad35 vector-mediated transduction on CHO cells expressing mutant CD46 lacking the cytoplasmic domain

To examine whether the intracellular domain of human CD46 is required for Ad35 infection, CHO transformants expressing human CD46 C2 isoforms lacking the cytoplasmic domain, CD46ΔCYT0 and CD46ΔCYT6, were transduced with Ad35L. All of the cytoplasmic domain is deleted in CD46ΔCYT0 (amino acid residues 347–369), while CD46ΔCYT6 contains the membrane-proximal 6 amino acids of the cytoplasmic tail and lacks a portion of the cytoplasmic domain (amino acid residues 352–369)

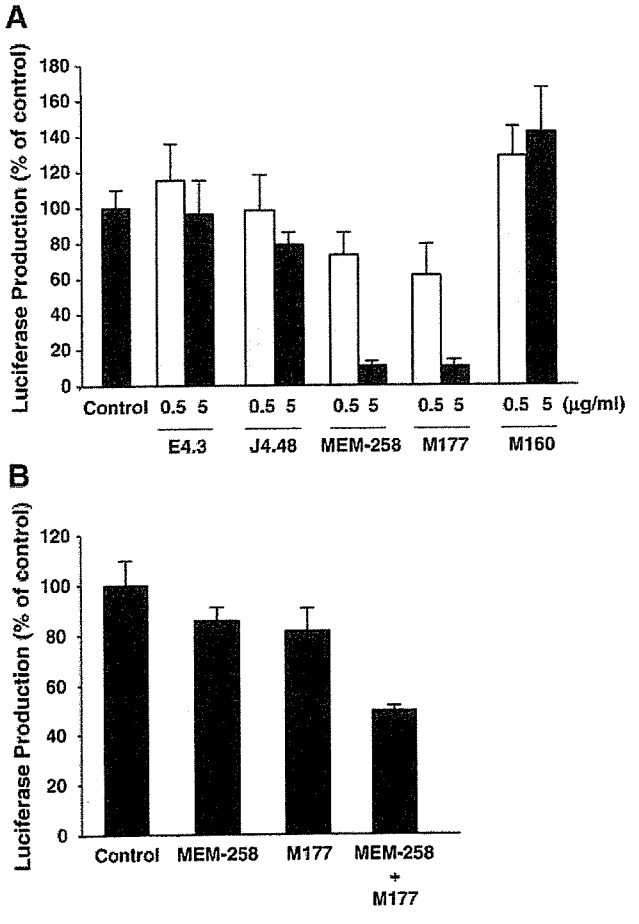

Fig. 5. Blocking of Ad35L-mediated transduction by monoclonal anti-CD46 antibodies. (A) Inhibition of Ad35L-mediated transduction by monoclonal anti-CD46 antibodies. E4.3, MEM-258, and J4-48 (recognizing SCR1), M177 (recognizing SCR2), and M160 (recognizing SCR3) were used as monoclonal anti-CD46 antibodies. CHO cells expressing wild-type CD46 were preincubated with each antibody at the indicated concentrations for 1 h and then infected with Ad35L at 3000 VP/cell. The luciferase productions in the cells were measured by luminescent assay 48 h after transduction. In control settings (Control), the cells were preincubated with medium only prior to transduction. The level of the luciferase production in control settings was almost the same as that in the presence of control mouse IgG (data not shown). (B) Combined inhibitory effect of MEM-258 and M177. The cells were preincubated with MEM-258 and/or M177 at 0.5 μg/ml. The transduction experiments were performed as described above. The data are expressed as the mean±S.D. (n=4).

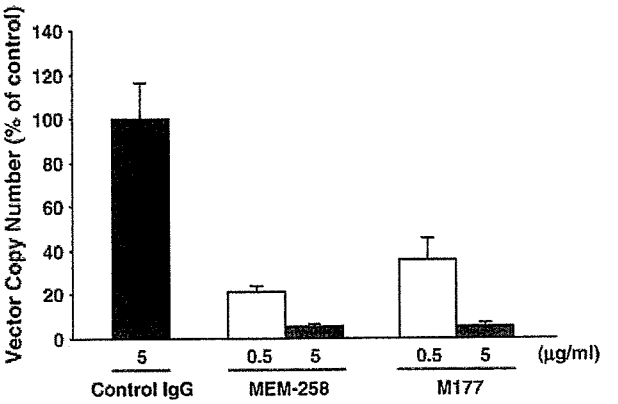

Fig. 6. Inhibition of cellular uptake of Ad35L by monoclonal anti-CD46 antibodies. CHO transformants expressing full-length CD46 were transduced with Ad35L in the presence of anti-CD46 antibody MEM-258 and M177 as described in Fig. 5. The total DNA, including the vector DNA, was extracted 48 h after transduction. The vector copy number was quantified by TaqMan-PCR. The data were normalized to the amounts of the vector DNA in CHO cells expressing full-length CD46 in the presence of control mouse IgG. The data are expressed as the mean±S.D. (n=4).

including the potential phosphorylation domain [26,27], which might be involved with various intracellular events, such as Ca^{2+} flux. The efficiency of the Ad35L-mediated transduction was similar between CHO cells expressing CD46ΔCYT6 and CHO cells expressing the full-length CD46 (Fig. 7). Furthermore, deletion of all the cytoplasmic domain significantly increased the transduction efficiency with Ad35L. These results indicate that the cytoplasmic domain of human CD46 would not be required to serve as a receptor for Ad35.

Next, we further measured the levels of CD46 expression in CHO transformants expressing wild-type CD46 or CD46ΔCYT0 following transduction with Ad35L to investigate why deletion of all the cytoplasmic domain increased the Ad35 vector-mediated transduction efficiency. The cytoplasmic domain is largely responsible to the downregulation of CD46 induced by MV [28], and

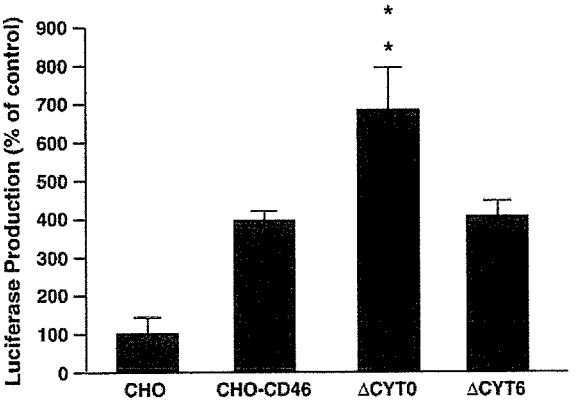

Fig. 7. Ad35L-mediated transduction in CHO cells expressing CD46 mutants lacking the cytoplasmic domain. The cells were transduced with Ad35L at 3000 VP/cells for 1.5 h. The luciferase productions in the cells were measured 48 h after transduction by luminescent assay. The data were normalized to the luciferase production in parental CHO cells. The data are expressed as the mean±S.D. (n=4). The asterisks indicate the level of significance (P<0.005 [double asterisk] for comparison with CHO-CD46).

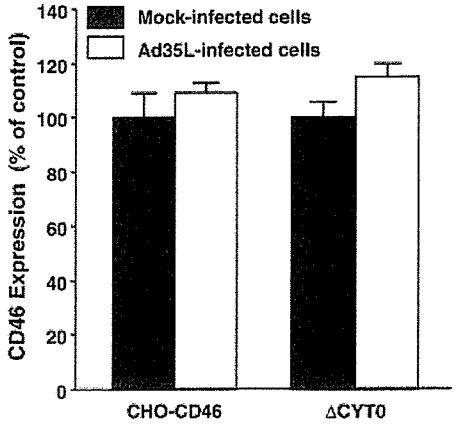

Fig. 8. CD46 expression levels in CHO transformants after infection with Ad35L. The CHO transformants expressing full-length CD46 or CD46ΔCYT0 were transduced with Ad35L at 3000 VP/cell for 1.5 h. After a 1.5-h incubation, the cells were subjected to flowcytometric analysis for measurement of CD46 expression. The data are expressed as the mean±S.D. (n=4).

CD46 downregulation might influence the infectivity of the viruses. Flowcytometric analysis demonstrated that CD46 downregulation did not occur in both CHO transformants expressing full-length CD46 or CD46ΔCYT0 following transduction with Ad35L (Fig. 8), suggesting that CD46 downregulation by Ad35 vectors was not involved in the increase in the transduction efficiencies of Ad35L in CHO cells expressing CD46ΔCYT0.

4. Discussion

Elucidation of the interaction between viruses and their receptors is of great importance for studies of virus pathogenicity. In addition, for the viruses that provide a framework for gene delivery vehicles, such information may help us not only to evaluate the transduction properties of virus vectors but also to improve virus vectors. In this study, CHO cells expressing CD46 deletion mutants and several monoclonal anti-CD46 antibodies were used to examine which regions are crucial for Ad35 infection. Infection experiments in cells expressing CD46 mutants lacking SCRs and blocking experiments using monoclonal anti-CD46 antibodies have already been used to determine the essential regions for infection of the pathogens recognizing CD46 in previous studies [16–18,29]. We applied this approach to elucidation of the crucial regions in CD46 for subgroup B Ad infection. The results presented herein demonstrated that the essential domains for Ad35 infection are located in SCR1 and 2, and that deletion of all the cytoplasmic domain in CD46 significantly increases Ad35 vector-mediated transduction.

Previous studies have demonstrated that MV binds to SCR1 and 2 [16,17], whereas infection of HHV6 is mediated by SCR2 and 3 [18]. Thus, SCR2 of CD46 is a crucial domain for all the human viruses utilizing CD46 (MV, HHV6, and subgroup B Ads). Moreover, SCR2-specific antibody M177 significantly inhibits the infection of all three viruses (Fig. 3) [18], suggesting that these viruses would interact with the region recognized by M177. The amino acids important for M177 binding, R69 and D70, which are located in the middle of SCR2 [30], are also present in CD46 of the cynomolgus monkey [31], which is susceptible to MV and HHV6. We also confirmed that primary cells isolated from the cynomolgus monkey were efficiently transduced with Ad35 vectors (data not shown).

Deletion of SCR1 as well as SCR2 largely decreased both the transduction efficiency and the cellular uptake of Ad35L (Figs. 3 and 4). However, SCR1-specific E4.3 and J4-48 did not significantly reduce the luciferase productions by Ad35L (Fig. 5). On the other hand, the antibody MEM-258, which also recognizes SCR1, significantly inhibited the Ad35 vector-mediated transduction and cellular uptake of Ad35L (Figs. 5 and 6). The amino acids important for binding of E4.3 and J4-48 are located on the top of SCR1 [31]. At present, it remains unclear where the epitope of MEM-258 is located within SCR1; however, the location of the epitope of MEM-258 would be different from those of E4.3 and J4-48, and would be important for Ad35 infection.

Recognition of SCR1 and 2 by Ad35 would be favorable for infection of Ad35. SCR1 and 2 are located on the upper region of CD46, leading to the decrease in electrostatic repulsion between the virus capsid and acidic cell surface proteins and the increase in attachment of Ad35 to the cell surface. Shayakhmetov and Lieber demonstrated that electrostatic repulsion between the virus capsid and cell surface is an important factor for Ad infection, especially for Ads possessing a short fiber shaft [32]. Ad35 has a shorter fiber shaft (9 nm) than Ad5 (37 nm).

During the preparation of this manuscript, two reports concerning the domains of human CD46 which interact with subgroup B Ads were published [25,33]. Gaggar et al. demonstrated that the subgroup B Ad-binding domain is located within SCR2 alone [33], while Fleischli et al. reported that the presence of both SCR1 and 2 is sufficient for infection of Ad35 and that binding of Ad35 is not confined to a single SCR domain [25]. Our data support the conclusion of Fleischli et al. The SCR2-specific antibody M177 and the deletion of SCR2 decreased Ad35 vector-mediated transduction (Figs. 3–6), suggesting that the region in SCR2 recognized by M177 would be important for interaction with Ad35. Luciferase production by Ad35L and cellular uptake of Ad35L in ΔSCR1 mutants was largely decreased, compared with CHO-CD46 cells (Figs. 3 and 4), suggesting that SCR1 would also play a role in Ad35 infection. Although the decrease in luciferase production and cellular uptake of Ad35L in the ΔSCR1 mutant might be due to conformational change of SCR2 by the deletion of SCR1, this is unlikely because the SCR2-specific antibody M177 showed positive staining in the ΔSCR1 mutant (Fig. 2). This suggests that the region recognized by M177 would hold an appropriate conformation in the ΔSCR1 mutant. Therefore, we conclude that both SCR1 and SCR2 are involved with Ad35 infection. The finding that the SCR1-specific antibody MEM-258 largely inhibited the transduction with Ad35L supports this conclusion (Figs. 5 and 6).

The cytoplasmic domain of human CD46 is not an absolute requirement in order for this protein to serve as an attachment receptor for Ad35 (Fig. 7). MV and HHV6 can also infect cells via mutant CD46 lacking the cytoplasmic domain [18,19]. However, the luciferase production was significantly increased in ΔCYT0, compared with that in CHO-CD46, in contrast, Ad35L mediated similar levels of luciferase productions in both CHO-CD46 and ΔCYT6. It remains unclear why the deletion of

the entire cytoplasmic domain of human CD46 increased the transduction efficiency, however, downregulation of CD46 was not observed in both CHO-CD46 and ΔCYT0 after transduction with Ad35L (Fig. 7). These results suggest that the increase in the transduction efficiencies of Ad35L in ΔCYT0 was not due to the lack of CD46 downregulation. One possibility for the increased transduction efficiencies in ΔCYT0 is that the amounts of CD46 which Ad35 vectors can access to would be increased by the deletion of the cytoplasmic domain. Maisner et al. demonstrated that CD46 are predominantly distributed in basolateral side of the cells and that CD46 lacking the entire cytoplasmic domain were transported to both apical and basolateral sides [34]. CD46ΔCYT0 might be more widely distributed than full-length CD46 in the CHO transformants, leading to the increase in the infection of Ad35 vectors. Another possibility is that the membrane-proximal 6 amino acids of the cytoplasmic domain in CD46 C2 isoform might contain a signal sequence for suppression of viral infection. Mouse macrophages expressing a tailless human CD46 mutant are more susceptible to MV infection than those expressing wild-type CD46 [35]. In addition to these functions of the cytoplasmic domain, the cytoplasmic domain plays important roles in immune responses through CD46, such as cytokine productions. Hirano et al. reported that the production of high levels of NO and IL-12 upon MV infection is dependent on the CD46 cytoplasmic domain [35]. Kurita-Taniguchi et al. demonstrated that intracellular phosphatase SHP-1 was found to be recruited to the cytoplasmic tail of human CD46 when human macrophages became sufficiently mature to produce IL-12 and NO in response to measles virus [36]. Therefore, the cytoplasmic domain might be involved with immune responses induced by Ad35 infection.

In summary, we demonstrated here that SCR1 and 2 of human CD46 are required for Ad35 infection, while the cytoplasmic domain of human CD46 is not crucial for an attachment receptor function for Ad35. These results offer insight into the interaction between human CD46 and subgroup B Ads, such as the internalization of Ad35 into the cells via CD46 and the crucial domain in the Ad35 fiber knob for binding to CD46.

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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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Keywords: *adenovirus serotype 35 vector; CD46; downregulation; fiber knob; peripheral blood mononuclear cells*

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.

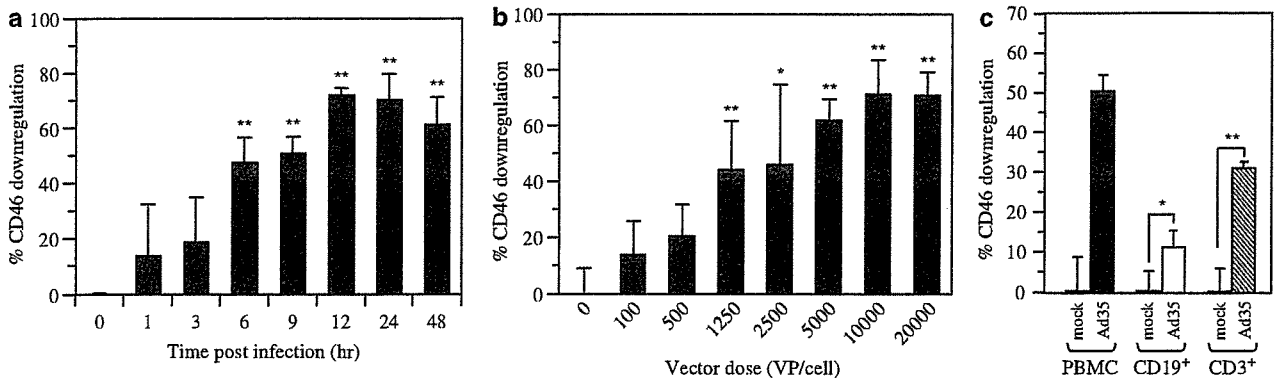

Figure 1 Downregulation of CD46 from the cell surface of PBMCs after infection by Ad35 vectors. (a) Time course of CD46 downregulation from the cell surface of PBMCs after infection with Ad35GFP. PBMCs were incubated with Ad35GFP at 10 000 VP/cell for up to 48 h. Cells were harvested at the indicated 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.

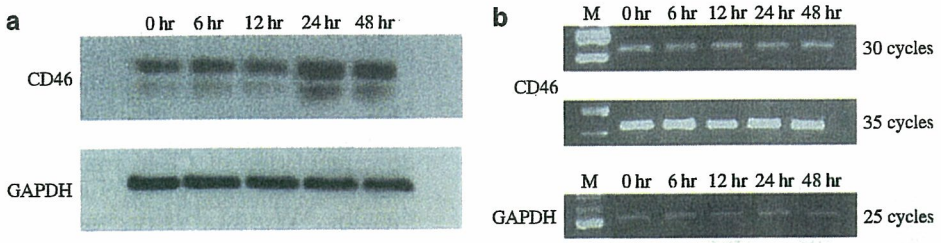

Figure 2 Total cellular protein levels and mRNA levels of CD46 following infection by Ad35 vectors. (a) Western blotting analysis for the total cellular protein levels of CD46 in PBMCs infected with Ad35GFP. PBMCs were incubated with Ad35GFP at 10 000 VP/cell for up to 48 h. Cells collected at the indicated 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

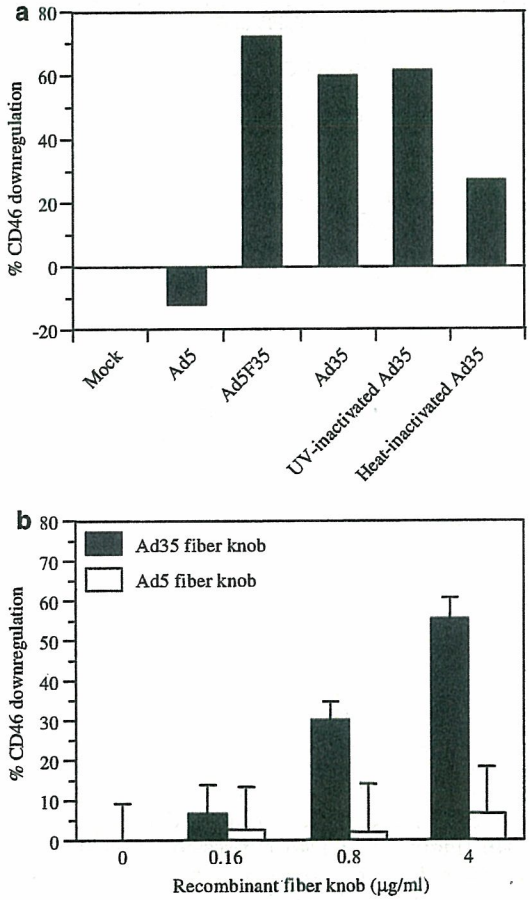

Figure 3 Role of Ad35 fiber knob protein on downregulation of surface CD46. (a) Downregulation of surface CD46 expression induced by various types of Ad vectors. PBMCs were incubated with Ad5GFP, Ad5F35GFP, Ad35GFP, UV-inactivated Ad35GFP or heat-inactivated Ad35GFP at 10 000 VP/cell for 24 h. After 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.

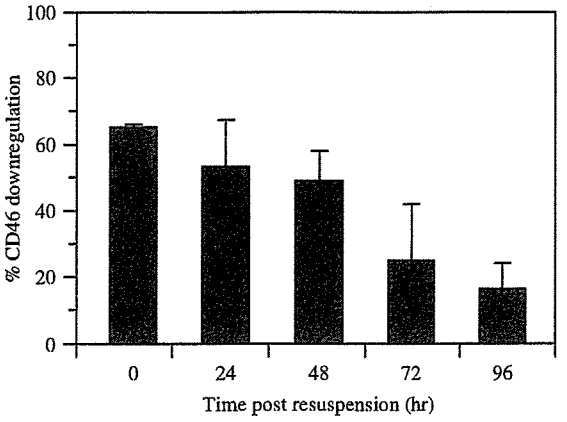

Figure 4 Recovery of surface CD46 expression after Ad35 vector-mediated downregulation. PBMCs were infected with Ad35GFP at 10 000 VP/cell for 24 h. After a 24-h infection, PBMCs were washed twice to remove the Ad35GFP, and resuspended and incubated in fresh medium. After incubation, cells were harvested 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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—Review—

35型アデノウイルスベクターの開発—遺伝子改変動物並びに霊長類を用いた検討—

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Characterization of Adenovirus Serotype 35 Vectors Using Genetically Modified Animals and Nonhuman Primates

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Recombinant Adenovirus (Ad) vectors are considered to be a promising gene delivery vehicle of high utility because they are easy to construct, can be produced at high titers, and efficiently transduce various types of cells. Ad vectors commonly used in the world, including clinical trials, are composed of Ad serotype 5 (Ad5), which belongs to subgroup C. In recent years, however, it has become apparent that Ad5 vectors have some drawbacks, such as high seroprevalence of anti-Ad5 antibodies in adults and low transduction efficiencies of Ad5 vectors in cells lacking a primary receptor for Ad5, coxsackievirus and adenovirus receptor (CAR). To overcome these limitations of Ad5 vectors, we have developed a novel type of Ad vector, which is composed of Ad serotype 35 (Ad35), belonging to subgroup B. Ad35 vectors recognize human CD46, not CAR, as a cellular receptor for infection. Human CD46 is expressed in almost all of human cells, leading to a broad tropism of Ad35 vectors to human cells, in contrast, expression of rodent CD46 is limited to the testis. Therefore, *in vivo* transduction properties of Ad35 vectors are not appropriately evaluated in normal mice. In order to evaluate the *in vivo* transduction properties of Ad35 vectors, Ad35 vectors were applied to human CD46-transgenic mice and nonhuman primates, which express CD46 in a similar pattern to humans. The data obtained using CD46-transgenic mice and nonhuman primates would provide valuable information towards clinical applications of Ad35 vectors.

Key words——adenovirus vectors; serotype; CD46; gene therapy

1. はじめに

遺伝子治療では遺伝子（核酸）が薬物（主剤）そのものであると考えられるが，通常の薬物とは異なり，多くの場合分子量 10^6 以上の巨大高分子である遺伝子を疾患部位の細胞の核にまで到達させる必要がある．したがって，遺伝子を細胞内，そして核内にまで送達するベクターが遺伝子治療の成否を決める極めて重要な要素であると言っても過言ではない．遺伝子導入用ベクターは，ウイルスを基本骨格としウイルス本来が兼ね備えている遺伝子送達メカニズムを利用したウイルスベクターと，脂質や高分子ポリマーを利用した非ウイルスベクターとに大別される．これまでウイルス・非ウイルスベクターを問わず多くの遺伝子導入用ベクターが開発されてきたが，なかでもアデノウイルス（Ad）ベクターは遺伝子導入用ベクターとして多くの長所を有することから，様々なアプローチからベクター改良研究が盛んに行われている．

Ad は軽い風邪を引き起こすウイルスの1つで，約 36 kb の直鎖状二本鎖 DNA をゲノムに持つエンベロープを持たないウイルスである．その形状は Fig. 1 に示すように，直径約 80 nm の正二十面体構造をしており，その頂点には感染に大きな役割を担っている 12 個のペントン（ファイバー及びペントンベース）と呼ばれる突起構造を持っている．Ad はこれまで多くの動物から単離されているが，ヒト Ad は現在までに 51 種類の血清型が同定されており，赤血球凝集活性の違いなどから A—F の 6 つの Subgroup に分類されている（Table 1）.[1] 現在汎用

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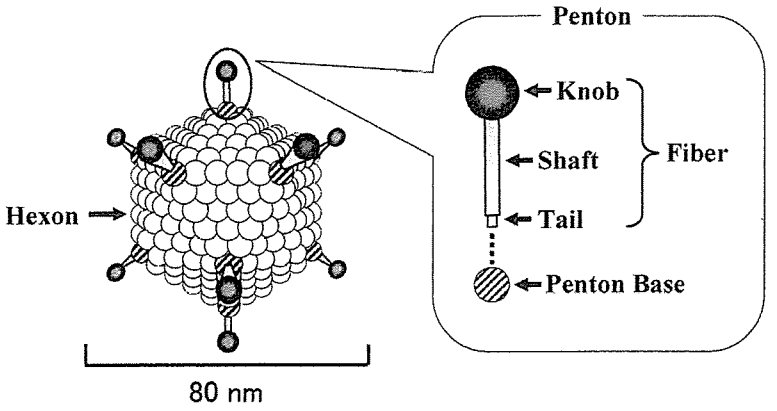

Fig. 1. A Schematic Diagram of Human Adenovirus
The double-stranded genomic DNA is packaged in the icosahedral particle with fibers projecting from the twelve vertices.

Table 1. Human Adenovirus Serotypes

Subgroup	Serotypes	Receptor*)
A	12, 18, 31	CAR
B	3, 7, 11, 14, 16, 21, 34, 35, 50	CD46
C	1, 2, 5, 6	CAR
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51	CAR
E	4	CAR
F	40, 41	CAR

CAR: coxsackievirus-adenovirus receptor. *) Some Ad serotypes recognize other receptors different from CAR and CD46.

されているAdベクターはSubgroup Cに属する5型Ad（若しくは2型）を基本骨格としている．5型Adベクターは全遺伝子治療臨床研究の約25%で用いられており（2006年1月現在），最近では遺伝子機能解析のためのツールとして基礎研究の場においても汎用されている．しかし近年，後述するように5型Adベクターの抱える様々な問題が明らかとなってきた．そこでわれわれは5型Adベクターの問題点を解決すべく，Subgroup Bに属する35型Adを基本骨格とした新規Adベクターを開発し，その機能解析を進めている．[2-6] 本稿では，われわれがこれまでに取り組んできた研究成果について紹介したいと思う．

2. 5型アデノウイルスベクターの問題点

1993年にアメリカにおいて嚢胞性繊維症に対して行われた5型Adベクターによる初めての臨床試験以降，[7] 5型Adベクターは癌や先天性遺伝子疾患などの臨床研究や多くの基礎研究に用いられてきた．これらの研究は5型Adベクターの有用性を示すと同時に，以下に示すような5型Adベクターが抱える問題点を明らかにした．

1）第一受容体であるCoxsackievirus and adenovirus receptor（CAR）の発現が低い細胞への遺伝子導入効率が低い．CARは1997年にBergelsonらによって2型及び5型Ad及びCoxsackie B virusの受容体として同定された分子量約46 KDaの膜タンパク質で，[8] 上皮細胞や肝細胞などで多く発現している．Subgroup Bに属するAdを除くほぼすべてのAdがCARを第一受容体としている．[9] したがって5型AdベクターはCAR陽性細胞に対しては効率よく感染し高い遺伝子導入効率を示すが，CAR陰性細胞では十分な遺伝子導入効率が得られない．CAR陰性細胞は意外にも多く，遺伝子治療の重要な標的細胞である造血幹細胞を始めとする血液細胞，血管平滑筋細胞，樹状細胞などがCAR陰性である．また癌細胞においては，癌の悪性度の進行に伴いCARの発現が低下することが報告されている．[10,11] さらに最近の研究ではCARがタイトジャンクションの形成に関与することが報告されており，[12,13] CAR陽性細胞においてもCARがタイトジャンクション部位に局在している場合には，立体障

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害により5型Adベクターが CARに到達できない可能性が指摘されている.

2) 既に多くの成人が5型Adに対する抗体を保持している. 5型Adは風邪の原因ウイルスの1つであることが知られており, 成人の多くは5型Adに対する抗体を既に有している. Seshidharらは, 成人の45—66%は5型Adに対する抗体を保持していると報告している.[14] 既存抗体は *in vivo* 遺伝子導入効率を大きく減弱させるだけでなく, 5型Adベクターの毒性を増強する可能性が指摘されている.[15] すなわち, 抗5型Ad抗体を保持しているヒトに5型Adベクターを投与した場合には, 抗体により遺伝子導入が阻害され十分な治療効果が得られないだけでなく, 大きな副作用を起こす危険性がある.

3. 35型アデノウイルスベクターの特徴

以上のような問題点を克服するため, われわれはSubgroup Bに属する35型Adを基本骨格とした新規Adベクターの開発を行った. 35型Adのベクター化に着目した理由としては (Fig. 2),

1) 受容体としてヒトCD46 (membrane cofactor protein) を認識して細胞に感染するため, 5型Adとは異なる感染域を示す. 35型Adを始めとするSubgroup Bに属するAdの受容体は長らく不明であった (われわれが35型Adベクターの開発に成功した時点においても不明であった). しかしながら35型Adが CAR以外の分子を受容体として認識すること,[9] 血球細胞に対し高い親和性を有すること[16]が既に明らかとなっていたことから, われわれは35型Adベクターが血液細胞を始めとして5型Adベクターでは遺伝子導入不可能な細胞に対しても効率よく感染するのではないかと考えた. 実際に開発した35型Adベクターの遺伝子導入特性を解析したところ, 35型Adベクターは CAR陽性細胞だけでなく, ヒトCD34陽性細胞を始めとするCAR陰性細胞に対しても高い遺伝子導入効率を示した.[2—4] その後2003年にヒトCD46が Subgroup B Adの受容体であることが報告されたが,[17,18] CD46はヒトではほぼすべての細胞で発現しており, 35型Adベクターの広い感染域を反映したものであった.

2) 35型Adに対する抗体を保持している成人の割合が低い. 先述のように成人の抗5型Ad抗体保持率は45%以上であるが, Subgroup B Adに対する抗体保持率は総じて低いことが報告されている. 特に35型Adに対する抗体保持率は20%以下と低いことから,[14,19] 35型Adベクターの遺伝子導入活性が既存抗体により阻害される可能性は低い. また35型Adは5型Adとは異なる Subgroupに属することから, 抗5型Ad抗体による阻害を受けない. われわれが抗5型Ad血清存在下における5型並びに35型Adベクターの遺伝子導入効率を検討した

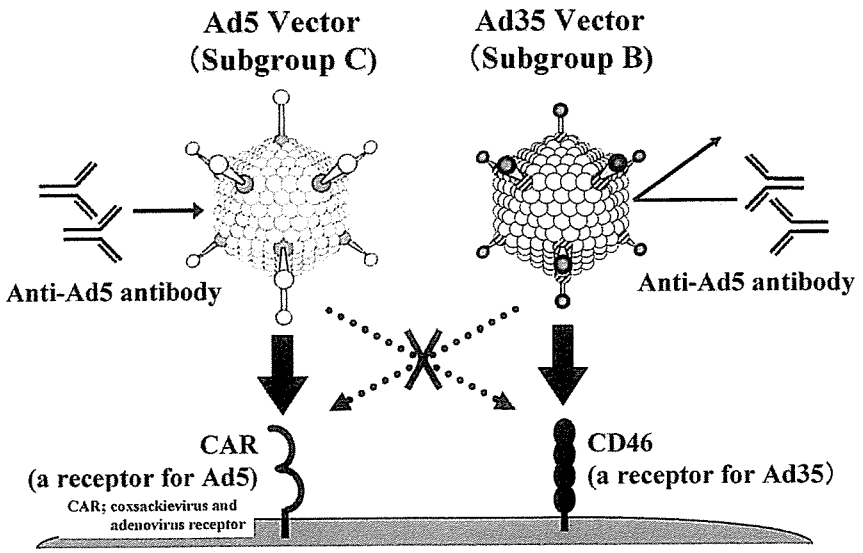

Fig. 2. A Schematic Diagram of Properties of Ad5 and Ad35 Vectors
Ad5 vectors infect cells via interaction with CAR (coxsackievirus and adenovirus receptor), on the other hand, Ad35 vectors recognize human CD46 for infection. Anti-Ad5 antibodies inhibit infection of Ad5 vectors, not Ad35 vectors.

ところ，5型Adベクターの遺伝子導入効率は抗5型Ad血清の濃度依存的に減少したが，35型Adベクターの遺伝子導入効率は影響を受けなかった．

一方で，ファイバータンパク質だけを35型Adなどの Subgroup B に属する Ad に由来するものに置換し，その他の領域は従来の5型Adから構成されたファイバー置換型5型Adベクターも開発されている．[20,21] ファイバーの先端部分であるノブ領域がCD46に直接結合する部位であることから，ファイバータンパク質のみを置換することで感染域を変えることが可能である．しかしほとんどの抗Ad中和抗体はヘキソン領域を認識するため，[22] ファイバー置換型5型Adベクターでは抗5型Ad抗体による阻害を回避することはできない．

4. CD46の特徴

Subgroup B Adの受容体であるCD46は，主に4つのisoform（BC1, BC2, C1, C2）が存在する分子量約55—65 KDaの糖タンパク質で，4つのShort consensus repeat (SCR), transmembrane domain, cytoplasmic tailなどから構成されている(Fig. 3)．CD46は本来，生体では補体成分であるC3bやC4bを分解することにより，自己の細胞を補体による攻撃から守る役割を担っている．またSubgroup B Adのみならず，麻疹ウイルス（一部のstrain），ヒトヘルペスウイルスtype 6, Nesseriaなども CD46を感染受容体としている．[23,24] これらの病原体についてはCD46のどの部位が感染に関与するのかが報告されており，Subgroup B Adについても先端領域に位置するSCR1及び2が感染に重要であることが明らかとなっている．[6,25] CARとは異なり，CD46はヒトでは血液細胞を始め，ほぼすべての細胞において発現しているのに対し（赤血球では発現していない），げっ歯類においてはCD46は精巣でしか発現していないこと，またマウスCD46はヒトCD46と比較してその相同性は約46%と低いことが知られている．[26] そのため35型Adベクターを通常のマウスに静脈内投与した場合の各臓器における遺伝子導入効率は，5型Adベクターと比較し極めて低いものであった．[3]

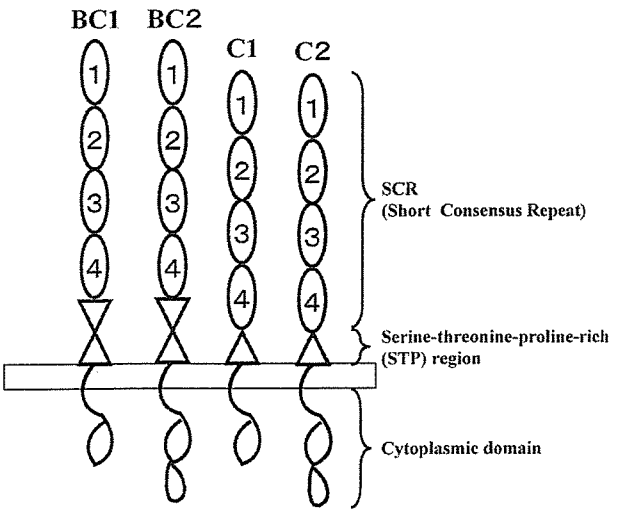

Fig. 3. A Schematic Diagram of Human CD46

Human CD46 is ubiquitously expressed in almost all human cells mainly as four isoforms (BC1, BC2, C1, C2) that are derived via alternative splicing. Human CD46 is composed of four cysteine-rich short consensus repeats (SCRs), a serine-threonine-proline-rich (STP) region, a short region of unknown function, a hydrophobic transmembrane domain, and a carboxy-terminal cytoplasmic domain.

5. CD46トランスジェニックマウスを用いた35型Adベクターの機能解析

そこでわれわれは，35型Adベクターが通常のマウスで遺伝子発現を示さないのは，受容体であるCD46が発現していないことが原因ではないかと考え，ヒトと同様にヒトCD46をほぼ全臓器で発現しているCD46トランスジェニック（CD46TG）マウス（大阪大学・岡部勝先生より供与）を用いて35型Adベクターの遺伝子導入特性を解析した．[5] まずCD46TGマウスにおけるCD46発現量をウエスタンブロットにて確認したところ，ヒトと同様にほぼすべての臓器でCD46の発現が確認された．次に35型Adベクターを野生型及びCD46TGマウスに静脈内及び腹腔内投与したところ，両投与経路ともにCD46TGマウスにおいて野生型マウスよりも有意に高い遺伝子導入効率が得られた（Fig. 4）．特に，両方の相同染色体にCD46遺伝子を有するホモCD46TGマウスの肝臓での遺伝子導入効率は，静脈内投与では野生型マウスの約10倍，腹腔内投与では約500倍高い値を示した．しかしながら，5型Adベクターと比較して，35型Adベクターによる遺伝子導入効率はCD46TGマウスにおいても依然低く，実験当初に期待していたような劇的な遺伝子導入効率の上昇はみられなかった．例えば35型Adベクターをホモ CD46TGマウスに静脈内投与したときの肝臓及び脾臓での遺伝子発現量は，5型Adベクターを野生型マウスに静脈内投与した場合のそれぞれ約20000分の1，及び50分の1であった．さらに35型AdベクターをCD46TGマウスに