Gene Therapy (2001) 8, 730–735

www.nature.com/gt

BRIEF COMMUNICATION

A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob

H Mizuguchi[1], N Koizumi[1,2], T Hosono[1], N Utoguchi[2], Y Watanabe[2], MA Kay[3] and T Hayakawa[1]
[1]*Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Tokyo;* [2]*Department of Pharmaceutics, Showa Pharmaceutical University, Tokyo, Japan; and* [3]*Departments of Pediatrics and Genetics, Stanford University School of Medicine, Stanford, CA, USA*

The use of recombinant adenovirus (Ad) vectors containing genetically modified capsid proteins is an attractive strategy for achieving targeted gene transfer. The HI loop of the fiber knob is a promising candidate location for the incorporation of foreign ligands for achieving this goal. However, the method of constructing an Ad vector containing a foreign ligand in the HI loop of the fiber knob has proved difficult. In this study, we developed a simple system to construct fiber-modified vectors. To do this, a vector plasmid containing a complete E1/E3-deleted Ad type 5 genome and a unique Csp45I and/or ClaI site between positions 32679 and 32680 of the Ad genome (residues threonine-546 and proline-547 of the fiber protein) was constructed. Oligonucleotides corresponding to the Arg-Gly-Asp (RGD) or Asn-Gly-Arg (NGR)-containing peptide motif (as a model) and containing a Csp45I and/or ClaI recognition site, were ligated into the Csp45I and/or ClaI-digested plasmid. The foreign transgene expression cassette was inserted into the E1 deletion site of the vector plasmid and the fiber-mutant Ad vector was produced by transfection of the PacI-digested plasmid into 293 cells. The virus containing the RGD or NGR peptide on the fiber knob was able to infect human glioma cells, which do not express coxsackievirus and adenovirus receptor (CAR), one of the Ad virus receptors, about 100–1000 times more efficient than the virus containing wild-type fiber. This suggested that the mutant virus mediated CAR-independent cell entry pathway. The simplicity of this method allows not only for easy construction of fiber-mutant Ad vectors, but also for screening of the peptides that target the vector to the desired cells and tissues. Gene Therapy (2001) **8,** 730–735.

Keywords: *gene therapy; adenovirus vector; fiber; retargeting*

Recombinant adenovirus (Ad) vectors are attractive vehicles for *in vitro* and *in vivo* gene transfer to a wide variety of cell types.[1,2] However, one of the limitations in the use of Ad vectors for gene transfer is the nonspecific tissue distribution after *in vivo* gene transfer and/or the inefficient gene transfer into cells lacking in one of the adenovirus receptors, coxsackievirus and adenovirus receptor (CAR).[3–6]

Ad infection requires two sequential steps. The first involves the attachment of the C-terminal knob domain of the fiber protein to the CAR on the cell surface.[3,7] Following binding, virus internalization via receptor-mediated endocytosis takes place through the interaction of the RGD motif of the penton bases with the secondary host cell receptors, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin.[8,9] The viral particle then escapes from the endosome and translocates to the nucleus.

Modification of fiber protein is an attractive strategy for altering the Ad tropism. Two approaches have been used for such modification. The first method is the addition of foreign peptides to the C-terminal end of the fiber knob,[10–13] while the second method involves the insertion of foreign peptides into the HI loop of the fiber knob.[14–16] In both systems, Ad tropism can be expanded by the binding of the foreign ligand to the cellular receptor, although true targeting cannot be obtained, because the native tropism is still maintained. Expanded tropism has been observed by use of mutant fiber proteins containing a stretch of lysine residues[10–13] or a RGD motif,[14,16] which target to the heparan sulfate or α_v integrin on the cellular surface, respectively. Based on several recent reports, the insertion of foreign peptides into the HI loop of the fiber protein appears to be more desirable than the addition of the peptides into the C-terminal portion of the protein, because modification of the C-terminal end sometimes prevents fiber trimerization and virus growth.[17] In addition, the C-terminus of the fiber points toward the virion,[18] and thus is not the optimal site for the addition of foreign ligand. In contrast, insertion of foreign peptides into the HI loop should not affect fiber trimerization[14,15] and leaves the peptides exposed on the outside of the virion.[15,19] However, the complexity of inserting foreign DNAs corresponding to the peptide of interest into this region of the fiber gene hampers the development of retargeted vectors. One study used a homologous recombination method in bacteria after the

Correspondence: H Mizuguchi, Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
Received 18 June 2000; accepted 6 February 2001

construction of a mutant fiber gene cloned in the plasmid to make such a vector.[14,15] However, this method required at least five steps, including transformation of the plasmid into different kinds of bacterial strains to produce the fiber-mutant Ad vector.

In this study, we developed a simple system that requires only a two-step, *in vitro* ligation to construct a fiber-mutant Ad vector containing the gene of interest. Two unique restriction sites, *Csp*45I and/or *Cla*I, were introduced into the sequence corresponding to the HI loop of the fiber gene that is cloned in the Ad vector plasmid with an E1/E3-deleted whole Ad genome. Therefore, the oligonucleotides corresponding to the foreign peptide of interest can be inserted into the HI loop of the fiber knob and a transgene containing vector created by this simple method.[20,21] As a proof-of-principle we demonstrate the creation and characteristics of an Ad vector with an insertion of a RGD peptide in the HI loop of the fiber knob and a luciferase expression cassette into the E1 deletion region. We also report the enhanced gene transfer activity of Ad vector containing the NGR peptide on the fiber knob.

The vector plasmids pAdHM15, -16, -17 and -18 were constructed as described below. pEco-ITR1,[21] which contains the right end of the Ad type 5 genome (from bp 27331 to the right end of the genome (Δ27865–30995)) was cut by *Apa*I and *Mun*I, and ligated with the *Apa*I/*Mun*I fragment of pBR-AM2, which is a pBR322-derived plasmid with an *Age*I and a *Mun*I site between the *Aat*II and *Bsa*I sites and a deletion between the *Pvu*II and *Bst*1107I sites. The resulting plasmid, pBR-AM3, contained the Ad genome (31905–32825). Then, four-piece ligation was performed using the *Apa*I/*Ase*I fragment of pBR-AM3, *Apa*I/*Bsm*AI fragment of pBR-AM3, *Bsa*AI fragment of pBR-AM3, and oligonucleotide 1 (5′-AACAGG AGACACAACTTCGAA*CATCGAT*CCAAGTGCATACTC TATGTCATTTTCATGGGACTGGTCTGGCCACAACTA-CAT-3′) and 2 (5′-TAATGTAGTTGTGGCCAGACCAGT CCCATGAAAATGACATAGAGTATGCACTTGG*ATCG ATG*TTCGAAGTTGTGTCTCC-3′; *Csp*45I or *Cla*I (Dam methylated) recognition sequences are underlined and italicized, respectively), resulting in pBR-AM4. pEco-AM4 were constructed by ligating the *Hpa*I/*Mun*I fragment of pBR-AM4 and the *Hpa*I/*Mun*I fragment of pEco-ITR5, the latter of which contains the right end of the Ad type 5 genome (from bp 27331 to the right end of the genome (Δ28133–30818)). Finally, pAdHM15 were constructed by the ligation of the *Srf*I/*Cla*I fragment of pAdHM2–1, which is a derivative of pAdHM2,[20] and the *Srf*/*Cla*I fragment of pEco-AM4, after which the *Cla*I site at the right end of the Ad genome was changed into a *Pac*I site by using oligonucleotides 3 (5′-CGTTAATTAA-3′) (*Pac*I recognition sequences are underlined). pAdHM16, -17 and -18 were constructed in the same manner. pAdHM15, -16, -17 and -18 have a complete E1/E3-deleted Ad genome with I-*Ceu*I, *Swa*I, and PI-*Sce*I sites in the E1 deletion region and a *Csp*45I and/or *Cla*I (Dam methylated) site between positions 32679 and 32680 of the Ad genome (residues threonine-546 and proline-547 of the fiber protein) as shown in Figure 1.

To construct a vector plasmid with a foreign oligonucleotide corresponding to the RGD-4C peptide, CDCGRDCFC, which binds with high affinities to integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) on the cell surface,[22–24] pAdHM15 was cut by *Csp*45I/*Cla*I and ligated with oligonucleotides 4 (5′-CGAAGTGTGACTGCCGCGGAGACTGTTTCTG-

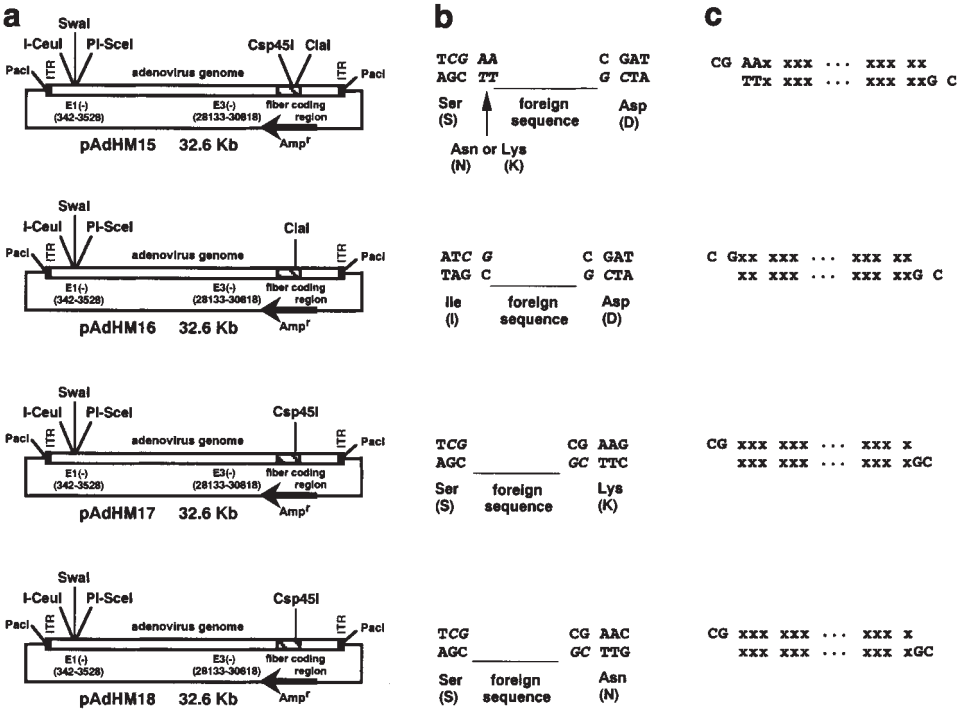

Figure 1 *Characterization of vector plasmids. (a) The vector plasmids, pAdHM15, -16, -17 and -18, contain an E1/E3-deleted Ad genome, Csp45I and/or ClaI site between positions 32679 and 32680 of the Ad genome (residues threonine-546 and proline-547 of the fiber protein), and the I-CeuI/SwaI/PI-SceI site in the E1 deletion region. (b) The sequence surrounding the site of insertion of foreign oligonucleotides for each vector plasmid is shown. The foreign oligonucleotides are italicized. (c) Oligonucleotides to be synthesized for each vector plasmid are shown. In pAdHM15, the oligonucleotides and the positive clones that are cut by Csp45I, but not by ClaI, are shown.*

3′) and 5 (5′-CGCAGAAACAGTCTCCGCGGCAGTCA-CACTT-3′). Next, ligated DNAs were transformed into DH5α, resulting in pAdHM15-RGD (Figure 2). The diagram in Figure 1 illustrates the sequences around the site of insertion of foreign oligonucleotides and the oligonucleotides that can be synthesized for each vector plasmid. Since *Csp*45I and *Cla*I produce compatible cohesive ends and the oligonucleotide can be inserted from either orientation, the oligonucleotide was designed so that the positive plasmid has a *Csp*45I site, but not a *Cla*I site (Figure 2). Both the self-ligated plasmid and the plasmid whose oligonucleotides are oriented in the opposite direction lack a *Csp*45I site. For this reason, the positive clone can be easily identified. Sequencing of the inserted oligonucleotides in pAdHM15-RGD verified that the clones contained the appropriate sequence.

Next, pAdHM15-RGD-CMVL2 was constructed by an *in vitro* ligation method[20,21] using I-*Ceu*I/PI-*Sce*I-digested pAdHM15-RGD and pCMVL1, which contains luciferase gene (derived from pGL3-Control (Promega, Madison, WI, USA)) in pHMCMV6.[21] I-*Ceu*I and PI-*Sce*I are rare-cutting enzymes that recognize at least 9, 10 and 11 bp, respectively.[25,26] As a control virus containing wild-type fiber protein, pAdHM4-CMVL2 was constructed by the ligation of pAdHM4 and pCMVL1.[20,21] Both pAdHM15-RGD-CMVL2 and pAdHM4-CMVL2 contained the cytomegalovirus (CMV) promoter-driven luciferase gene and the bovine growth hormone (BGH) polyadenylation signal.

To prepare the virus, pAdHM15-RGD-CMVL2 and pAdHM4-CMVL2 were digested with *Pac*I and purified by phenol–chloroform extraction and ethanol precipitation. Linearized pAdHM15-RGD-CMVL2 and pAdHM4-CMVL2 were transfected into 293 cells. Viruses (AdRGD-L2 and Ad-L2) were prepared as described previously[20] (Figure 2) and purified by $CsCl_2$ step gradient

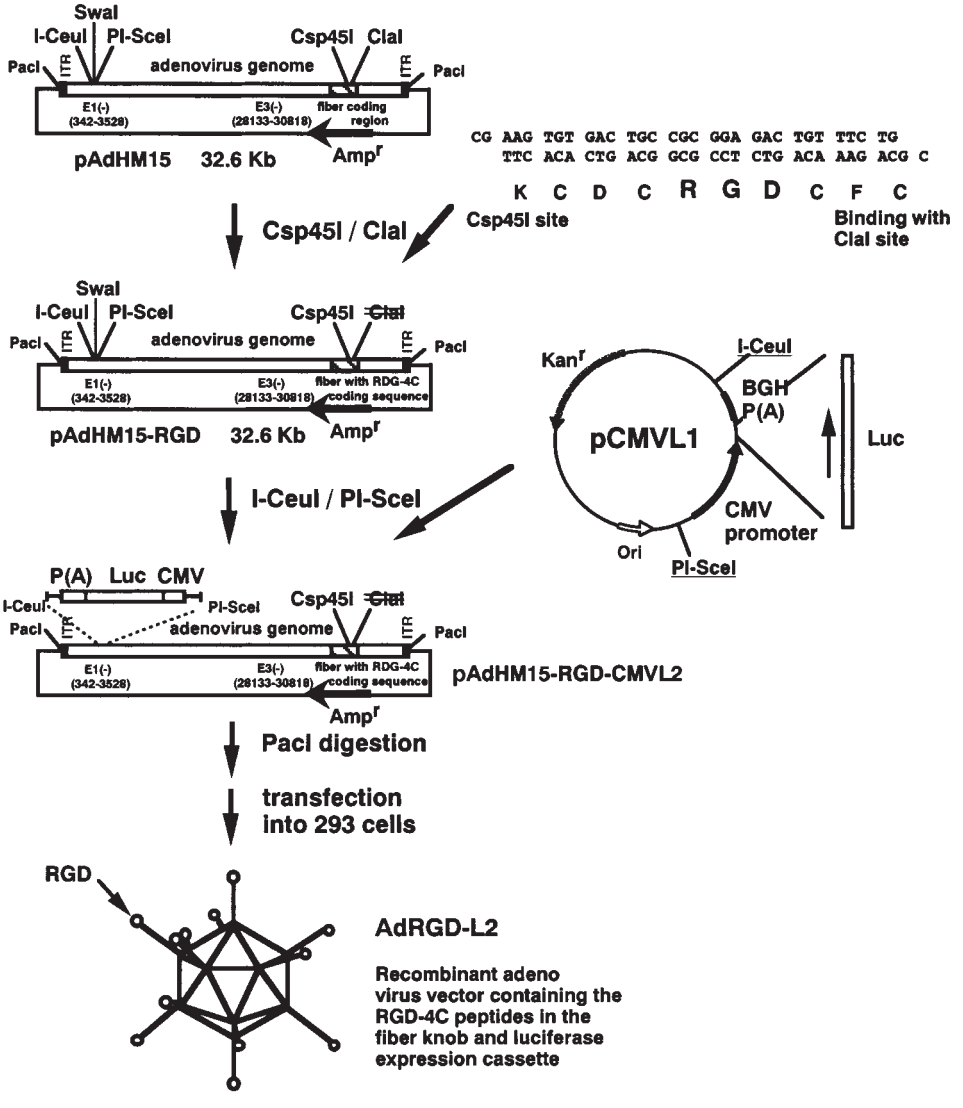

Figure 2 *The construction strategy for the Ad vector containing the RGD-4C peptide in the HI loop of its fiber knob and a luciferase expression cassette in the E1 deletion region. pAdHM15 was cut by Csp45I/ClaI, and ligated with the oligonucleotides corresponding to the RGD-4C peptide, resulting in pAdHM15-RGD. The oligonucleotides were designed such that the positive clone had a Csp45I site, but not a ClaI site for convenience of selection. Then, the luciferase expression cassette was inserted into the E1 deletion region of pAdHM15-RGD by an in vitro ligation method using I-CeuI and PI-SceI (pAdHM15-RGD-CMVL2) (refs 20, 21). The Ad vector containing the RGD-4C peptide in the HI-loop of its fiber knob and the luciferase expression cassette (AdRGD-L2) were produced by the transfection of the PacI-digested plasmid into 293 cells.*

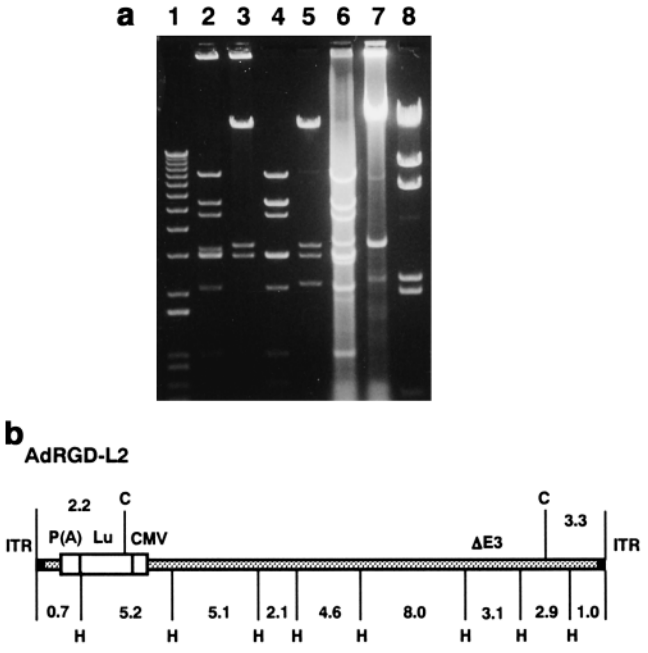

Figure 3 *Restriction endonuclease analysis. (a) The vector plasmid (pAdHM15-RGD or pAdHM15-RGD-CMVL2) or the luciferase-expressing recombinant adenovirus DNA with the RGD-4C peptide in the fiber knob (AdRGD-L2) was digested with HindIII/PacI, Csp45I/PacI, HindIII or Csp45I, and electrophoresed on 0.7% of agarose gel. Lane 1, 1 kb DNA ladder marker; lane 2, HindIII/PacI-digested pAdHM15-RGD; lane 3, Csp45I/PacI-digested pAdHM15-RGD; lane 4, HindIII/PacI-digested pAdHM15-RGD-CMVL2; lane 5, Csp45I/PacI-digested pAdHM15-RGD-CMVL2; lane 6, HindIII-digested AdRGD-L2 viral DNA; lane 7, Csp45I-digested AdRGD-L2 viral DNA; lane 8, HindIII-digested lambda DNA marker. A faint band of about 3.2 kb of HindIII-digested AdRGD-L2 viral DNA (lane 6) is nonspecific, because XhoI-digested AdRGD-L2 viral DNA only contained the expected fragments (not shown). (b) HindIII and Csp45I restriction map of the recombinant adenoviral DNAs (AdRGD-L2). Fragment sizes in kilobases are shown below and uper the genome. CMV, cytomegalovirus intermediate–early promoter/enhancer; P(A), bovine growth hormone polyadenylation signal.*

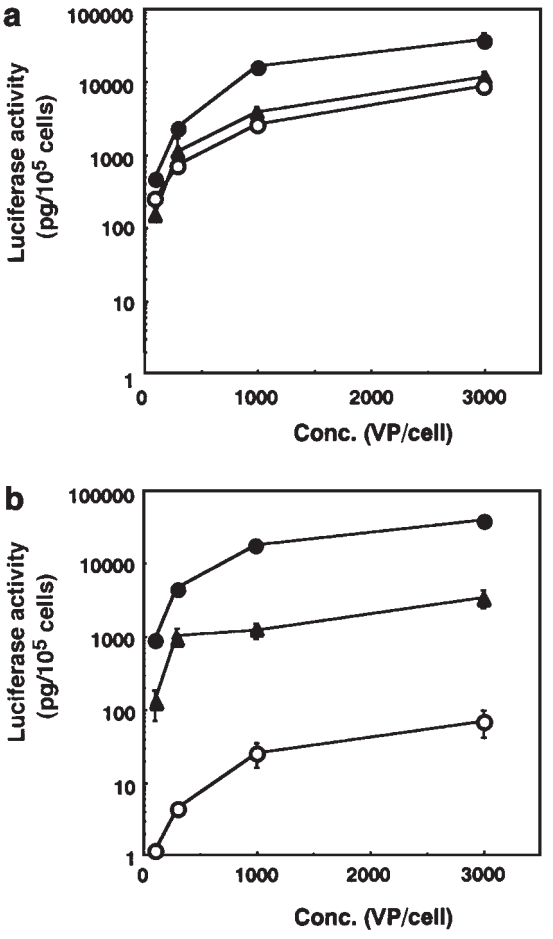

Figure 4 *Comparison of luciferase expression in cultured cells transduced by AdRGD-L2 and Ad-L2. SK HEP-1 (a) and LN444 (b) cells (1×10^4 cells) were transduced with Ad-L2 (open circle), AdRGD-L2 (closed circle), or AdNGR-L2 (closed triangle) (100, 300, 1000, 3000 vector particles (VP) per cell). Two days later, the luciferase expression was determined. All data represent the mean ± s.d. of four experiments.*

ultra-centrifugation followed by $CsCl_2$ linear gradient ultra-centrifugation. Determination of virus particle titer was accomplished spectrophotometrically by the method of Maizel *et al*.[27] The plaque-forming unit to particle ratio of the virus was 1:23 for AdRGD-L2 and 1:8 for Ad-L2. DNA restriction analysis showed that the vector plasmid with or without the luciferase expression cassette and viral DNA contained the expected fragments (Figure 3).

Dmitriev *et al*[14] first developed an Ad vector having an RGD-4C peptide, CDCRGDCFC, incorporated into the HI loop of its fiber knob, and demonstrated that this virus was capable of mediating a RGD-integrin-dependent, CAR-independent, gene delivery. The only difference between the fiber contained in our vector (AdRGD-L2) and that contained in their vector is that AdRGD-L2 has three additional amino acids flanking the RGD-4C peptide (RGD-4C, SKCDCRGDCFCD – additional three amino acids are underlined) due to the additional nucleotides contained within the *Csp*45I and *Cla*I recognition sites.

To examine the functionality of the RGD-4C peptide in the fiber of AdRGD-L2, SK HEP-1[28] and LN444 cells[29] were transduced with AdRGD-L2 or Ad-L2. Two days later, luciferase expression was determined by a luciferase assay system (PicaGene LT2.0, Toyo Inki Co. Ltd, Tokyo, Japan) (Figure 4). The RGD-4C peptide binds with high affinities to both $\alpha_v\beta_3$ and $\alpha_v\beta_5$.[24] Therefore, the level of CAR and integrin ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) expression in these cells were also examined by flow cytometry with a Cyto ACE-150 Auto Cell Screener (JASCO, Tokyo, Japan) (Figure 5). The luciferase enzymatic activity following transduction with AdRGD-L2 and Ad-L2 was only four to eight times different in SK HEP-1 cells. In contrast, LN444 cells transduced with AdRGD-L2 and Ad-L2 resulted in approximately a 1000-fold difference in detectable luciferase activity (Figure 4). LN444 cells expressed integrins ($\alpha_v\beta_5$) but expressed no or only small amounts of CAR, while SK HEP-1 cells expressed both CAR and integrins ($\alpha_v\beta_5$) (Figure 5). Thus, the presence of the RGD-4C peptide in the fiber knob was required for efficient transduction of these CAR-deficient cells. These results suggested that AdRGD-L2 infected CAR-deficient cells via an RGD integrin-dependent, CAR-independent cell entry pathway in a manner similar to that of the vector developed by Dmitriev *et al*.[14]

We also constructed an Ad vector having an NGR peptide, CNGRCVSGCAGRC, on the fiber knob. Luciferase expressing the Ad vector containing the NGR peptide, AdNGR-L2, was created by the same method as that used

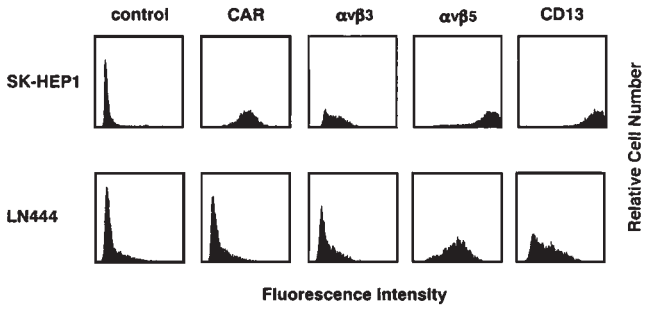

Figure 5 *Flow cytometry analysis of levels of CAR and integrin ($\alpha_v\beta_3$, $\alpha_v\beta_5$) and CD13 expression in SK HEP-1 and LN444 cells. The cells (5×10^5 cells) were labeled with mouse monoclonal antibody RmcB (kindly provided by Dr JM Bergelson, The Children's Hospital of Philadelphia, PA, USA) for human CAR detection, mouse anti-human integrin $\alpha_v\beta_3$ (LM609; Chemicon International, Temecula, CA, USA), mouse anti-human integrin $\alpha_v\beta_5$ (P1F6; Gibco BRL, Gaithersburg, MD, USA), and mouse anti-human CD13 (WM15; PharMingen, San Diego, CA, USA). The cells were then incubated with FITC-conjugated goat anti-mouse IgG second antibody (PharMingen), and were analyzed by flow cytometry.*

for AdRGD-L2 (plaque-forming unit to particle ratio of the virus was 1:30 for AdNGR-L2). Oligonucleotides 6 (5′-CGGCTGCAACGGCCGCTGCGTGAGCGGCTGCGC CGGCCGCTG-3′) and 7 (5′-CGCAGCGGCCGGCGCAGC CGCTCACGCAGCGGCCGTTGCAGC-3′) were inserted into the *Cla*I site of pAdHM15. This peptide sequence was originally isolated as a tumor-forming peptide by a phage display library and recognized different cellular receptors from the RGD-4C peptide.[22] Aminopeptidase N (CD13) has recently been isolated as a molecule recognized by the NGR peptide.[30] We examined the expression levels of CD13 on SK HEP-1 and LN444 cells and the transduction efficiency of AdNGR-L2 (Figures 4 and 5). As shown in Figure 4, AdNGR-L2 mediated luciferase expression at levels approximately 100 times higher in CAR-deficient LN444 cells expressing moderate amounts of CD13 than Ad-L2, although the activity was less than that with AdRGD-L2. In contrast, SK HEP-1 cells transduced with AdNGR-L2 and Ad-L2 resulted in similar levels (1.5 to two times difference) of detectable luciferase activity. Thus, AdNGR-L2 infected the cells via both CAR-dependent and CAR-independent (NGR-CD13-dependent) cell entry pathways. This is the first report showing the enhanced gene delivery by the Ad vector containing the NGR peptide on the fiber knob. As the NGR peptide has a higher affinity to tumor vasculature,[22] Ad vector containing the NGR peptide could be useful for anti-angiogenic gene therapy.

Recently, various peptides capable of binding to specific tissues *in vivo*, such as tumor and brain have been discovered by using phage display libraries.[22,23,31–33] Inclusion of these peptides, which are recovered by the selected phage in the fiber of the Ad vector may direct the vector to desired tissues and cells at higher affinities than a vector containing wild-type fiber. The simplicity of the method described here would allow rapid and easy screening of various Ad vectors with peptide ligands for targeted delivery, although the vectors containing foreign peptide in the HI loop of the fiber knob still have the binding activity with its native receptor, CAR.

We constructed various vector plasmids (pAdHM15, -16, -17 and -18; Figure 1a) that contain different sequences at the *Csp*45I and/or *Cla*I recognition sites to vary the additional few amino acids that are included when cloning in new fiber peptides. For example, pAdHM16 has *Ile* (I) and *Asp* (D) as shown in Figure 1b. The viruses derived from pAdHM16, -17 and -18 have only two additional amino acids. In the case of screening a large number of foreign peptides, it is desirable that the method for producing fiber-mutant Ad vectors be as simple as possible. If the gene of interest to be inserted into the E1 deletion region does not have a *Csp*45I and/or *Cla*I site and the vector plasmid containing gene of interest is used, various Ad plasmids containing both foreign peptides in the fiber and gene of interest can be prepared in a one-step ligation reaction. The vector plasmid containing the gene of interest is cut by *Csp*45I and/or *Cla*I, after which oligonucleotides are introduced into the fiber-coding region. In this way, various fiber-mutant Ad vectors containing the gene of interest can be produced with minimal labor.

Recently, systems for the propagation of Ad vectors with genetically modified receptor specificities were reported.[34–36] In these systems, packaging cell lines expressing pseudoreceptors were developed to produce the targeted Ad vector with a pseudoligand. The system described here, combined with such packaging cell lines containing new ligands for vector binding in the absence of CAR,[36,37] should be a powerful tool for the construction of targeted recombinant Ad vectors for gene therapy.

Acknowledgements

We would like to thank Jun Murai and Nobuko Heishi for technical assistance. We would like to thank Dr M Tada (Hokkaido University, Japan) and Dr JM Bergelson (The Children's Hospital of Philadelphia, PA) for kindly providing LN444 cells and mouse monoclonal antibody RmcB, respectively. This work was supported by grants from the Ministry of Health and Welfare in Japan and Grant-in-Aid for Scientific Research on Priority Areas (C). MAK was supported by NIH DK49022.

References

1 Kay MA, Woo SL. Gene therapy for metabolic disorders. *Trends Genet* 1994; **19**: 253–257.
2 Kozarsky KF, Wilson JM. Gene therapy: adenovirus vectors. *Curr Opin Genet Dev* 1993; **3**: 499–503.
3 Bergelson JM *et al.* Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 1997; **275**: 1320–1323.
4 Miller CR *et al.* Differential susceptibility of primary and established human glioma cells to adenovirus infection: targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. *Cancer Res* 1998; **58**: 5738–5748.
5 Pickles RJ *et al.* Limited entry of adenovirus vectors into well-differentiated airway epithelium is responsible for inefficient gene transfer. *J Virol* 1998; **72**: 6014–6023.
6 Zabner J *et al.* Lack of high affinity fiber receptor activity explains the resistance of ciliated airway epithelia to adenovirus infection. *J Clin Invest* 1997; **100**: 1144–1149.
7 Henry LJ *et al.* Characterization of the knob domain of the adenovirus type 5 fiber protein expressed in *Escherichia coli*. *J Virol* 1994; **68**: 5239–5246.
8 Bai M, Harfe B, Freimuth P. Mutations that alter an Arg-Gly-Asp (RGD) sequence in the adenovirus type 2 penton base protein abolish its cell-rounding activity and delay virus reproduction in flat cells. *J Virol* 1993; **67**: 5198–5205.
9 Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins avb3 and avb5 promote adenovirus internalization but not virus attachment. *Cell* 1993; **73**: 309–319.

10 Bouri K *et al*. Poly-lysine modification of adenoviral fiber protein enhances muscle cell transduction. *Hum Gene Ther* 1999; **10**: 1633–1640.
11 Gonzalez R *et al*. Increased gene transfer in acute myeloid leukemic cells by an adenovirus vector containing a modified fiber protein. *Gene Therapy* 1999; **6**: 314–320.
12 Wickham TJ *et al*. Increased *in vitro* and *in vivo* gene transfer by adenovirus vectors containing chimeric fiber proteins. *J Virol* 1997; **71**: 8221–8229.
13 Yoshida Y *et al*. Generation of fiber-mutant recombinant adenoviruses for gene therapy of malignant glioma. *Hum Gene Ther* 1998; **9**: 2503–2515.
14 Dmitriev I *et al*. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus. *J Virol* 1998; **72**: 9706–9713.
15 Krasnykh V *et al*. Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. *J Virol* 1998; **72**: 1844–1852.
16 Reynolds PN, Dmitriev I, Curiel DT. insertion of an RDG motif into the HI loop of adenovirus fiber protein alters the distribution of transgene expression of the systemically administered vector. *Gene Therapy* 1999; **6**: 1336–1339.
17 Hong JS, Engler JA. Domains required for assembly of adenovirus type 2 fiber trimers. *J Virol* 1996; **70**: 7071–7078.
18 Xia D, Henry LJ, Gerard RD, Deisenhofer J. Crystal structure of the receptor-binding domain of adenovirus type 5 fiber protein at 1.7 A resolution. *Structure* 1994; **2**: 1259–1270.
19 Xia D, Henry L, Gerard RD, Deisenhofer J. Structure of the receptor binding domain of adenovirus type 5 fiber protein. *Curr Top Microbiol Immunol* 1995; **199**: 39–46.
20 Mizuguchi H, Kay MA. Efficient construction of a recombinant adenovirus vector by an improved *in vitro* ligation method. *Hum Gene Ther* 1998; **9**: 2577–2583.
21 Mizuguchi H, Kay MA. A simple method for constructing E1 and E1/E4 deleted recombinant adenovirus vector. *Hum Gene Ther* 1999; **10**: 2013–2017.
22 Arap W, Pasqualini R, Ruoslahti E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science* 1998; **279**: 377–380.
23 Pasqualini R, Koivunen E, Ruoslahti E. Alpha v integrins as receptors for tumor targeting by circulating ligands. *Nat Biotechnol* 1997; **15**: 542–546.
24 Koivunen E, Wang B, Ruoslahti E. Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins. *Biotechnology* 1995; **13**: 265–270.
25 Gimble FS, Thorner J. Homing of a DNA endonuclease gene by meiotic gene conversion in *Saccharomyces cerevisiae*. *Nature* 1992; **357**: 301–306.
26 Marshall P, Lemieux C. Cleavage pattern of the homing endonuclease encoded by the fifth intron in the chloroplast large subunit rRNA-encoding gene of *Chlamydomonas eugametos*. *Gene* 1991; **104**: 241–245.
27 Maizel JVJ, White DO, Scharff MD. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 1968; **36**: 115–125.
28 Heffelfinger SC *et al*. SK HEP-1: a human cell line of endothelial origin. *In Vitro Cell Dev Biol* 1992; **28A**: 136–142.
29 Asaoka K *et al*. Dependence of efficient adenoviral gene delivery in malignant glioma cells on the expression levels of the Coxsackievirus and adenovirus receptor. *J Neurosurg* 2000; **92**: 1002–1008.
30 Pasqualini R *et al*. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. *Cancer Res* 2000; **60**: 722–727.
31 Barry MA, Dower WJ, Johnston SA. Toward cell-targeting gene therapy vectors: selection of cell-binding peptides from random peptide-presenting phage libraries. *Nat Med* 1996; **2**: 299–305.
32 Koivunen E *et al*. Tumor targeting with a selective gelatinase inhibitor. *Nat Biotechnol* 1999; **17**: 768–774.
33 Pasqualini R, Ruoslahti E. Organ targeting *in vivo* using phage display peptide libraries. *Nature* 1996; **380**: 364–366.
34 Douglas JT *et al*. A system for the propagation of adenoviral vectors with genetically modified receptor specificities. *Nat Biotechnol* 1999; **17**: 470–475.
35 Einfeld DA *et al*. Construction of a pseudoreceptor that mediates transduction by adenoviruses expressing a ligand in fiber or penton base. *J Virol* 1999; **73**: 9130–9136.
36 Roelvink PW *et al*. Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae. *Science* 1999; **286**: 1568–1571.
37 Kirby I *et al*. Mutations in the DG loop of adenovirus type 5 fiber knob protein abolish high-affinity binding to its cellular receptor CAR. *J Virol* 1999; **73**: 9508–9514.