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Preparation of a Claudin-Targeting Molecule Using a C-Terminal Fragment of *Clostridium perfringens* Enterotoxin

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ABSTRACT

Although most malignant tumors are epithelia-derived carcinomas, methods for specific and effective delivery of antitumor agents to carcinomas have not been developed. Recent reports indicate that epithelia overexpress claudin-3 and -4, which are integral membrane proteins of epithelial tight junctions. This suggests that claudins can be targeted for tumor therapy, but there is not currently a method for delivering drugs to claudin-expressing cells. In the present study, we evaluated whether a potent claudin-4-binding C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) would allow targeting to claudin-4-expressing cells. We fused C-CPE to the protein synthesis inhibitory factor (PSIF), which lacks the cell binding domain of *Pseudomonas* exotoxin. This fusion protein, C-CPE-PSIF, was cytotoxic to MCF-7 human breast cancer cells, which express endogenous claudin-4, but it was not toxic to mouse fibroblast L cells, which lack endogenous claudin-4. The cytotoxicity of C-CPE-PSIF was attenuated by pretreating the MCF-7 cells with C-CPE but not bovine serum albumin. Also, deletion of the claudin-4-binding region of C-CPE reduced the cytotoxicity of C-CPE-PSIF. Finally, we found that C-CPE-PSIF is toxic to L cells expressing claudin-4 but not to normal L cells or cells expressing claudin-1, -2, or -5. These results indicate that use of the C-CPE peptide may provide a novel way to target drugs to claudin-expressing cells.

Epithelial-derived tumors account for 90% of all malignant tumors, and their resistance to chemotherapy is a major clinical problem (Greenlee et al., 2000). Recent progress in combinatorial chemistry, proteomics, and genomics research has further advanced the development of effective drugs against carcinomas, but, for clinical application, it is also essential to develop selective and efficient drug delivery systems for these novel drugs (Allen and Cullis, 2004).

These drugs can be delivered by targeting several cell surface molecules, including carcinoembryonal antigen, carboanhydrage IX, and epithelial cell adhesion molecule (Steffens et al., 1997; Chester et al., 2000; Mayer et al., 2000; McLaughlin et al., 2001). In fact, experimental therapies have been developed using antibody-mediated targeting of these molecules. However, because carcinoembryonal antigen and carboanhydrage IX are expressed by carcinomas as well as normal epithelium in kidney and liver, side effects are difficult to avoid (Steffens et al., 1997; Chester et al., 2000; Mayer et al., 2000). Moreover, the antibody for epithelial cell adhesion molecule itself is toxic to normal epithelium (McLaughlin et al., 2001).

Tight junctions (TJs), which are points of intercellular contact and interaction, are characteristic and complex structures in the epithelia. TJs play a critical role in forming a barrier between apical and basal sides of the cell, and they are present on the lateral side of the cell where they mediate intercellular interactions (Schneeberger and Lynch, 2004). Loss of polarity is a typical feature of transformation in epithelial cells. Furthermore, abnormal localization of membrane proteins, including TJ components, adherence junction proteins, and apical and basal proteins, is observed during carcinogenesis (Wodarz, 2000; Yarden and Sliwkowski, 2001; Vermeer et al., 2003). These findings indicate that abnor-

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ABBREVIATIONS: TJ, tight junction; CPE, *C. perfringens* enterotoxin; C-CPE, C-terminal fragment of *C. perfringens* enterotoxin; PSIF, protein synthesis inhibitory factor derived from *Pseudomonas* exotoxin; PE, *Pseudomonas* exotoxin; C-CPE-PSIF, C-terminal fragment of *C. perfringens* enterotoxin fused to a protein synthesis inhibitory factor; BSA, bovine serum albumin; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; LDH, L-lactate dehydrogenase.

mally localized membrane proteins may be useful for targeting drugs to carcinoma cells.

Claudin is an approximately 23-kDa transmembrane protein found in the TJ, and it plays a pivotal role in the barrier function of the TJ (Tsukita et al., 2001). There are more than 20 members of claudin family, and they are expressed in a tissue-specific manner (Morita et al., 1999a,b). For instance, claudin-1 is ubiquitously expressed, and claudin-3 is observed in the lung and liver. In mice, claudin-5 is expressed in all blood endothelial cells. Claudin-6 is widely expressed only in the fetus. Interestingly, the overexpression of claudins is frequently observed in the epithelium of ovarian cancer, hepatocellular carcinoma, malignant pancreatic cancer, and prostate cancer (Hough et al., 2000; Long et al., 2001; Michl et al., 2003; Rangel et al., 2003; Cheung et al., 2005). Therefore, claudins are promising candidates for the targeting of anticancer drugs to carcinoma cells.

Clostridium perfringens enterotoxin (CPE) is a single polypeptide with a molecular mass of 35 kDa that causes food poisoning associated with most human food-borne illnesses (McClane and Chakrabarti, 2004). CPE is made up of two functionally distinct domains: an approximately 22-kDa N-terminal domain that mediates cytotoxicity and an approximately 13-kDa C-terminal domain (C-CPE) that mediates binding (McClane and Chakrabarti, 2004). Claudin-3 and -4 are the receptors for CPE (Katahira et al., 1997; Sonoda et al., 1999), and we and others have shown that they bind to CPE via the C-CPE domain (Katahira et al., 1997; Sonoda et al., 1999; Fujita et al., 2000; Kondoh et al., 2005). These findings suggest that CPE could be used for the targeting of claudins on epithelial carcinoma cells. Indeed, CPE has been successfully used to treat human ovarian and pancreatic cancers, both of which express high levels of claudin-3 or -4 (Michl et al., 2001; Santin et al., 2005).

In the present study, we investigated whether C-CPE can be used to target claudin-4-expressing cells. For this purpose, we utilized a PSIF derived from PE as a reporter molecule (Leamon et al., 1993; Mesri et al., 1994; Beers et al., 2000) to assess targeting of claudin-4-expressing cells. PE (GenBank accession no. K01397; http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=151215) binds to the cell surface and is internalized via the endocytotic pathway, after which it escapes from endosomes into the cytosol. The PE fragments (PSIF) released into the cytosol inhibit protein synthesis by blocking the function of elongation factor 2 (Ogata et al., 1990). The PSIF, which cannot invade into cells, does not show any cytotoxicity because of lacking the cell binding domain. We show here that a C-CPE-PSIF fusion was toxic to cells expressing claudin-4 but not to cells expressing claudin-1. In addition, the cytotoxic effects of C-CPE-PSIF were dose dependently attenuated by C-CPE. Thus, C-CPE is a potent molecule for targeting of claudin-4-expressing cells and should be useful as a system for delivering drugs against malignant carcinomas.

Materials and Methods

Chemicals. Anti-FLAG M2 affinity gel, anti-FLAG M2 monoclonal antibody-peroxidase conjugate, FLAG peptide, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Glutathione-Sepharose 4B resin, thrombin protease, and Benzamidine-Sepharose 4 Fast Flow were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Horseradish peroxidase-labeled Ab, anti-claudin-1 pAb, anti-claudin-2 pAb, anti-claudin-4 mAb, anti-claudin-5 mAb, and anti β-actin mAb were obtained from Zymed Laboratories (South San Francisco, CA). All other reagents used were research grade.

Cell Culture. Human breast cancer cell line MCF-7 cells and human intestinal cell line Caco-2 cells were maintained in RPMI 1640 medium and Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37°C, respectively. Mouse fibroblast cell line L cells and mouse claudin-expressing L cells, kindly provided by Dr. S. Tsukita (Kyoto University, Japan) (Morita et al., 1999b; Sonoda et al., 1999), were cultured in modified Eagle's medium containing 10% fetal calf serum at 37°C.

Preparation of C-CPE-PSIF Fusions. The plasmids containing fusions of PSIF with C-CPE and C-CPE lacking its C-terminal 30 amino acids (C-CPE289) were prepared as follows. C-CPE and C-CPE289 were amplified by polymerase chain reaction (PCR) using pET16bHis$_{10}$C-CPE as a template (Katahira et al., 1997), a common forward primer (5′-CCATGGCCGAGAGATGTGTTTTAACAGTT-3′, NcoI site is underlined), and a reverse primer for C-CPE (5′-GCGGCCGCAAATTTTTGAAATAATATTGA-3′, NotI site is underlined) or C-CPE289 (5′-GCGGCCGCTATATCAACATAATGATCTTT-3′, NotI site is underlined). The resulting PCR fragments were subcloned into the pGEM T-Easy Vector to create pTA/C-CPE and pTA/C-CPE289 (Promega, Madison, WI), and the sequences were confirmed. PSIF was amplified using PSIF primer-1 (5′-GATGATCGATCGCGGCCGCAGGTGCGCCGGTGCCGTATCCGGATCCGCTGGAACCGCGTGCCGCA*GACTACAAAGACGACGACGACAAA*CCCGAGGGCGGCAGCCTGGCCGCGCTGACC-3′, the underline indicates the NotI site, and the italic letters indicate the FLAG sequence), PSIF primer-2 (5′-GATCGATCGATCACTAGTCTACAGTTCGTCTTTCTTCAGGTCCTCGCGCGGCGGTTTGCCGGG-3′, the underline indicates SpeI site), and *Pseudomonas aerginosa* cDNA as a template. The resulting PCR products were subcloned into the pGEM T-Easy Vector, and the sequences were confirmed. The NotI/SpeI-digested PSIF fragment was inserted into the NotI/SpeI-digested pY02 (Yamamoto et al., 2003) to generate pY02-PSIF. The pTA/C-CPE and pTA/C-CPE289 plasmids were digested with NotI and NcoI, and the fragments were inserted into NotI/NcoI-digested pY02-PSIF to generate pY02-C-CPE-PSIF and pY02-C-CPE289-PSIF. The C-CPE-PSIF plasmids were transduced into *Escherichia coli* strain TG1, after which the cells were grown at 37°C in 2YT medium (Invitrogen, Carlsbad, CA) containing 2% glucose to an optical density at 600 nm of 0.6 to 0.9. The medium was then changed to 2YT medium containing 1 mM isopropyl β-D-thiogalactopyranoside. After an additional 18 h of culture at 30°C, the cells were harvested and centrifuged. The resulting supernatant was applied to anti-FLAG M2 affinity gel, and the bound proteins were eluted with FLAG peptide. The buffer was exchanged with phosphate-buffered saline (PBS) using a PD-10 column (GE Healthcare), and the purified protein was stored at −80°C until use. Purification of the of C-CPE-PSIF proteins was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE), followed by staining with Coomassie Brilliant Blue and by immunoblotting with anti-FLAG M2 antibody (Fig. 1; data not shown). Protein was quantified using a commercially available assay kit with BSA as a standard (Bio-Rad, Hercules, CA).

Preparation of C-CPE. Following digestion of pTA/C-CPE with XhoI and NotI, the resulting DNA fragments were inserted into XhoI/NotI-digested pGEX4T-1 (GE Healthcare). Glutathione *S*-transferase-fused C-CPE was prepared as follows. The pGEX4T-1 plasmid encoding C-CPE was transduced into *E. coli* BL21 (DE3), after which the cells were cultured in LB medium at 37°C until the logarithmic phase. The culture was then adjusted to 1 mM isopropyl β-D-thiogalactopyranoside, and the cells were grown for an additional 6 h. The cells were harvested and then solubilized in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA, pH 8.0) containing 100 μg/ml lysozyme, 5 mM dithiothreitol, and 1.5% *N*-lauroylsarcosine. The lysate was centrifuged, after which the super-

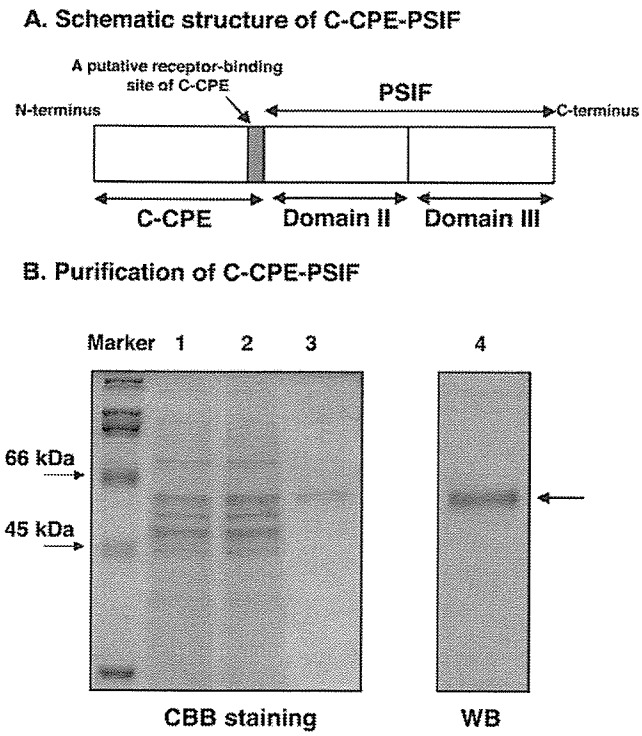

Fig. 1. Preparation of C-CPE-PSIF. A, schematic structure of C-CPE-PSIF. C-CPE-PSIF is a fusion of C-CPE and PSIF. The putative receptor-binding region of C-CPE is located in its C terminus and is shown here as the dark area (Hanna et al., 1991). PSIF contains domains II and III of PE. Domain II is critical for escape of the toxin from the endosome to the cytosol, and domain III is responsible for inhibition of protein synthesis (Ogata et al., 1990). B, purification of C-CPE-PSIF. C-CPE-PSIF was expressed in *E. coli* and isolated by anti-FLAG affinity chromatography. The purification of C-CPE-PSIF was confirmed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB) (lanes 1–3) and by immunoblotting using an antibody against FLAG (lane 4). The arrow indicates the purified C-CPE-PSIF. Lane 1, *E. coli* lysates; lane 2, flow-through; lanes 3 and 4, eluted fraction.

natant was collected and adjusted to 2% Triton X-100. The supernatant was incubated with glutathione-agarose beads for 2 h at 4°C. The beads were then washed with the lysis buffer, and C-CPE was eluted from the beads by cleavage with thrombin. Thrombin was removed from the eluted protein using Benzamidine-Sepharose 4 Fast Flow. The buffer was then exchanged with PBS using a PD-10 column. The purification of C-CPE was confirmed by SDS-PAGE (data not shown).

Trypan Blue Assay. Cells were treated with vehicle or C-CPE-PSIF proteins for the indicated periods. Both the floating and adherent cells were recovered and were suspended in ice-cold PBS. The cell suspension was adjusted to 0.2% trypan blue, and the number of stained (dying or dead) and unstained (living) cells was counted. At least 300 cells were counted to determine the fraction of dead cells.

L-Lactate Dehydrogenase Release Assay. The release of lactate dehydrogenase (LDH) from the cells was analyzed using a CytoTox96 NonRadioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's protocol. LDH release was calculated using the following equation: percentage of maximal LDH release = LDH in the cultured medium/total LDH in the culture dish.

Competition Assay. MCF-7 cells were pretreated with C-CPE or BSA at the indicated concentration for 1 h, after which C-CPE-PSIF was added to the cells. After an additional 36 h of culture, LDH release was assayed as described above.

Statistical Analysis. Statistical significance of differences was assessed using one-way analysis of variance followed by Dunnett's test. Differences were considered significant when $p < 0.05$.

Results

Preparation of C-CPE-PSIF. To assess the ability of C-CPE to target claudin-4-expressing cells, we fused it with PSIF. Because of limitations of the restriction sites in the PSIF-encoding plasmid, we linked C-CPE to a site upstream of the 5′ terminus of PSIF (Fig. 1A). As shown in Fig. 1B, C-CPE-PSIF was produced effectively by *E. coli* and could be purified by affinity chromatography using anti-FLAG antibodies. Its molecular size as determined by SDS-PAGE was identical to its predicted size (58 kDa).

Cytotoxic Properties of C-CPE-PSIF. To examine the cytotoxic properties and specificity of C-CPE-PSIF, we compared its effects on L cells, which lack endogenous claudins, and MCF-7 cells, which express endogenous claudin-4 (Fig. 2A). Trypan blue dye exclusion showed C-CPE-PSIF caused

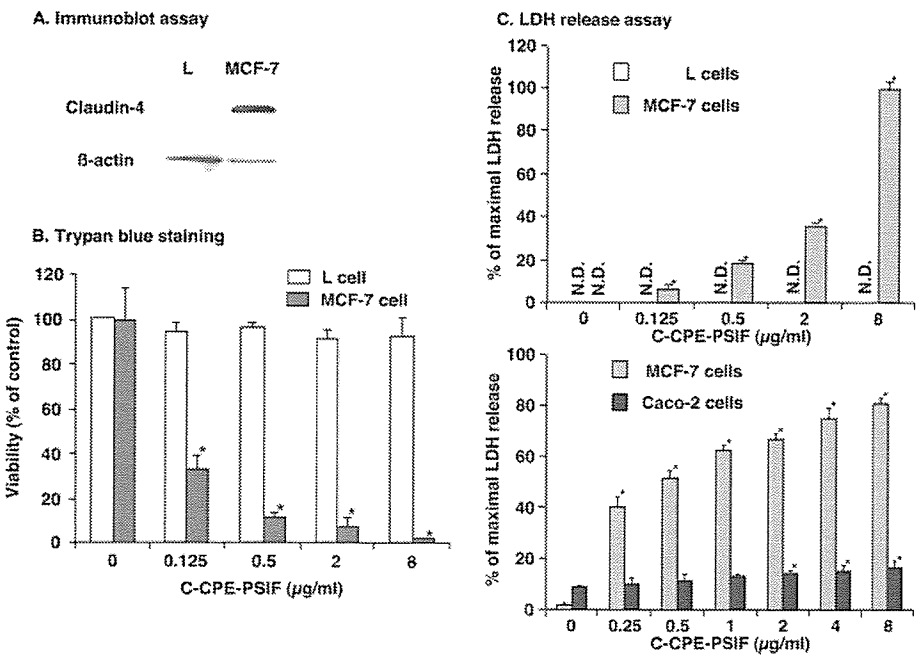

Fig. 2. Cytotoxicity of C-CPE-PSIF. A, expression of claudin-4 in L and MCF-7 cells. Claudin-4 expression was assessed by immunoblotting. Blots were also probed with a β-actin antibody to show equivalent loading. B, trypan blue assay. The cells were treated with C-CPE-PSIF at the indicated concentrations for 36 h. The cells were then harvested and stained with 0.2% trypan blue. The blue-stained cells (dead and dying cells) and the unstained cells (living cells) were counted under a microscope. At least 300 cells were counted in each condition. Results indicate the means ± S.D. ($n = 3$). *, significantly different from the vehicle-treated cells ($P < 0.05$). C, LDH release assay. Cells were treated for 36 h with the indicated concentrations of C-CPE-PSIF, after which the rate of LDH release was determined. There was no LDH release from the C-CPE-PSIF-treated L cells. Results represent means ± S.D. ($n = 3$). *, significantly different from the vehicle-treated cells ($P < 0.05$). N.D., not detected.

dose-dependent cell death in MCF-7 cells, with 92% death after a 36-h treatment with 2 μg/ml C-CPE-PSIF (Fig. 2B). Similar results were observed in the LDH release assay. In this case, 8 μg/ml C-CPE-PSIF caused the release of approximately 100% of the LDH (Fig. 2C). In contrast, even at 8 μg/ml, C-CPE/PSIF was not cytotoxic to L cells and Caco-2 cells (Fig. 2, B and C). Taken together, these results suggested that C-CPE-PSIF is toxic to claudin-4-expressing tumor epithelial cells.

Targeting Properties of C-CPE-PSIF. We next used a competition assay to determine whether C-CPE-PSIF binds to MCF-7 cells via C-CPE. As shown in Fig. 3A, pretreatment of the cells with C-CPE dose dependently attenuated the cytotoxic activity of 0.5 μg/ml C-CPE-PSIF, with a maximal effect observed at 5 μg/ml C-CPE. In contrast, pretreatment of the cells with 10 μg/ml BSA did not reduce the cytotoxicity of 0.5 μg/ml C-CPE/PSIF. These results suggest that C-CPE-PSIF interacts with MCF-7 cells via the C-CPE domain.

C-CPE is known to bind to claudin-4 via its 30 C-terminal acids (Kondoh et al., 2005). To confirm that claudin-4 is involved in the cytotoxicity of C-CPE-PSIF, we prepared C-CPE289-PSIF, which lacks the claudin-4-binding region of C-CPE. As expected, C-CPE-PSIF had a powerful toxic effect on MCF-7 cells, reaching 70% cell death at 4 μg/ml. In contrast, even at 8 μg/ml, C-CPE289-PSIF was not cytotoxic. These results suggested that interaction with claudin-4 is essential for the cytotoxicity of C-CPE-PSIF.

To confirm this possibility, we evaluated the cytotoxic activity of C-CPE-PSIF in L cells expressing claudin-1 (CL1/L cells), -2 (CL2/L cells), -4 (CL4/L cells), and -5 (CL5/L cells) (Figs. 4, B and C). C-CPE-PSIF was not cytotoxic in CL1/L, CL2/L, and CL5/L cells (Fig. 4C), and it was toxic in CL4/L cells (31.3 and 73.5% LDH release at 0.1 and 5 μg/ml, respectively). These results confirm that the C-CPE domain of C-CPE-PSIF interacts with claudin-4 on the cell membrane.

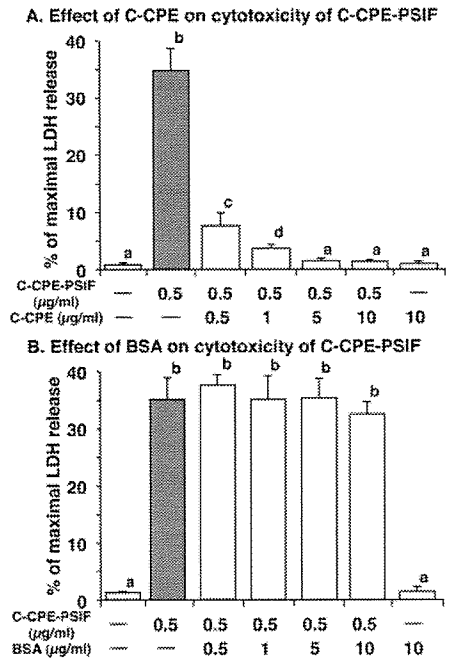

Fig. 3. C-CPE-PSIF interacts with cells via the C-CPE domain. MCF-7 cells were treated with the indicated concentrations of C-CPE (A) or BSA (B) for 1 h. The cells were then treated for 36 h with 0.5 μg/ml C-CPE-PSIF, after which LDH release was assessed. Results represent the means ± S.D. (n = 3). There were significant differences between conditions with the different superscripts ($P < 0.05$).

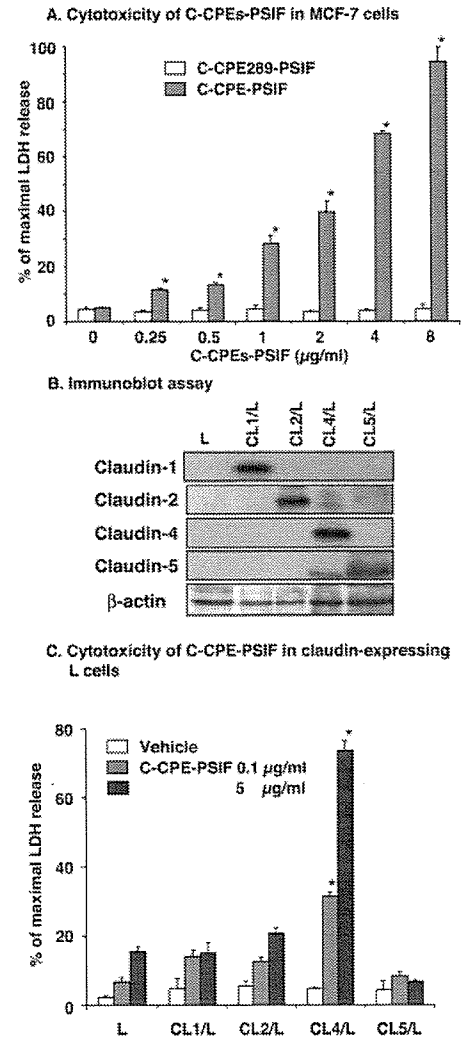

Fig. 4. Involvement of claudin-4 in the cytotoxicity of C-CPE-PSIF. A, cytotoxicity of C-CPE289-PSIF in MCF-7 cells. MCF-7 cells were treated for 36 h with the indicated concentrations of C-CPE289-PSIF or C-CPE-PSIF, after which LDH release was assessed. The results represent the means ± S.D. (n = 3). *, significantly different from the vehicle-treated cells ($P < 0.05$). B, immunoblot analysis of claudin-expressing L cells. The expression profiles for claudin family members were determined by immunoblotting. C, cytotoxicity of C-CPE-PSIF in claudin-expressing L cells. L cells expressing claudins were treated for 36 h with the indicated concentrations of C-CPE-PSIF, after which LDH release was assessed. Results represent the means ± S.D. (n = 3). *, significantly different from the L cells ($P < 0.05$).

Discussion

In the current studies, we found that a C-CPE-PSIF fusion protein is potently cytotoxic to claudin-4-expressing cells. This suggests that C-CPE could be used to confer claudin binding to drugs.

We used a fusion in which the C terminus of C-CPE was linked to the N terminus of PSIF. Thus, the receptor-binding region of C-CPE may be influenced by fusion with PSIF. To investigate this further, we are currently attempting to prepare C-CPE-PSIF with a G4S linker inserted between the C-CPE and PSIF domains. In the current studies, it was necessary to connect PSIF to the C terminus of C-CPE be-

cause of technical aspects of plasmid construction, and we need to consider the function of C-CPE when preparing other claudin-4-targeting molecules. Solution of the three-dimensional structures of claudins and CPE should be helpful. Competitive analysis using C-CPE revealed that C-CPE-PSIF interacted with cells via the C-CPE domain. Thus, cellular uptake of C-CPE-PSIF appears to be dependent on the interaction of claudin-4 with C-CPE. Indeed, we found that L cells expressing claudin-1, -2, or -5 are insensitive to C-CPE-PSIF and that deletion of the claudin-4-binding domain of C-CPE eliminates its cytotoxicity. These results indicate that C-CPE-PSIF binds to claudin-4 on the cell surface. Similarly, Fujita et al. (2000) showed that CPE binds to claudin-4 but not claudin-1, -2, or -5. Also, expression of claudin-4 but not -1 or -5 confers sensitivity to CPE (Sonoda et al., 1999; Fujita et al., 2000). Thus, our data are consistent with previous findings, and they show that fusion with C-CPE, the receptor-binding region of CPE, confers claudin binding to PSIF. The receptor-binding region of CPE was previously narrowed to the C-terminal 30 amino acids (Hanna et al., 1991). Similarly, we reported that deletion of the C-terminal 30 amino acids of C-CPE eliminates its ability to bind claudin-4 (Kondoh et al., 2005). Here, we showed that deletion of the 30 amino acids in C-CPE-PSIF abolishes its toxic effects. Taken together, the results indicate that C-CPE could be used to target drugs to claudin-4.

For the use of claudins as targets for drug delivery, it is important to understand whether molecules binding to claudin on the cell surface are taken up into the cells. PSIF is a useful as a reporter for screening ligands (Siegall et al., 1990; Theuer et al., 1992; Leamon et al., 1993). Because C-CPE-PSIF was cytotoxic in claudin-4-expressing cells, we expect that C-CPE-PSIF must enter the cytosol. This leaves the question of how C-CPE-PSIF enters the cells. One possibility is that it is taken up by endocytosis, after which it escapes from the endosomes into the cytosol. Generally, proteins are targeted to clathrin-coated vesicles by a sorting signal sequence, including YXXØ or EXXXLL (where X is any amino acid, and Ø is a bulky hydrophobic residue) (Bonifacino and Traub, 2003). Because claudin-4 contains an ALGVLL motif at amino acids 92 to 97 and a YVGW motif at amino acids 165 to 168 (Ivanov et al., 2004), it may be taken up by clathrin-mediated endocytosis. Indeed, Matsuda et al. (2004) showed that the endocytosis of claudins occurs during the remodeling of TJs.

Claudins are overexpressed in some tumor cells. Administration of CPE has been shown to reduce the growth of claudin-4-overexpressing human ovarian and pancreatic tumors (Michl et al., 2001; Rangel et al., 2003; Santin et al., 2005). CPE contains not only a claudin-4-binding domain but also a cytotoxic domain (McClane and Chakrabarti, 2004). Therefore, it is hard to use CPE in itself as a targeting molecule to claudin-4. Offner et al. (2005) reported that antibodies for claudins bind to claudin-expressing carcinomas, suggesting that anti-claudin antibodies or their F_v domains could be used to target antitumor agents to claudin-positive tumors. However, targeting of an antitumor agent to cells via a claudin has never been achieved. In this point, C-CPE is a useful claudin-4-targeting molecule, and C-CPE could target not only antitumor agents but also liposomes to claudin-4-overexpressing tumor cells. Although claudin-4 is also distributed in normal tissues such as normal colon epithelium and several glands, the expression in normal tissues is weaker than in tumors (Long et al., 2001; Michl and Gress, 2004). Therefore, detailed analysis for mechanism of interaction between C-CPE and claudin-4 is needed for a future application of antitumor therapy using C-CPE. In the present study, we found that C-CPE-PSIF was nontoxic in Caco-2 cells. We previously reported that addition of C-CPE in basal side not apical side of Caco-2 monolayer resulted in decrease of the barrier function of TJ (Masuyama et al., 2005; Takahashi et al., 2005). McClane and Chakrabarti (2004) also reported that Caco-2 cells are more sensitive to CPE when CPE is applied to their basal side than when CPE is applied to their apical sides. Taken together, insensitivity of Caco-2 cells to C-CPE-PSIF may be due to polarization of Caco-2 cells. This is an interesting founding, and it is an important issue to clarify the relationship between sensitivity of C-CPE-PSIF and polarization of claudin-4 in tumor tissues and normal tissues. C-CPE reduced the barrier function of TJ of epithelia (Kondoh et al., 2005), and utilization of C-CPE may facilitate drug delivery to intratumor tissues.

We previously showed that the C-terminal 16 amino acids of C-CPE are responsible for its interaction of claudin-4 (Kondoh et al., 2005), indicating that modification of the C terminus of C-CPE could allow other claudins to be targeted. For example, it may be useful to target claudin-10 because it is overexpressed in hepatocellular carcinomas (Cheung et al., 2005). Because the plasmid encoding C-CPE-PSIF is a phagemid vector, its binding specificity can be easily manipulated using phage display. We are currently attempting to identify the precise claudin-4 binding region of C-CPE and to use a phage display library to prepare versions of C-CPE that can bind other claudins.

In summary, we showed that the C-CPE domain of C-CPE-PSIF targets claudin-4. This is the first report that C-CPE can allow the targeting of a drug to claudin-4. Because of these results, we are currently developing a claudin-targeting drug delivery system.

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Design and synthesis of a Tat-related gene transporter: A tool for carrying the adenovirus vector into cells

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Abstract—A Tat-related peptide, acetyl-Gly-Arg-Arg-Arg-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Gly-Cys amide, designed to transport an Adenovirus vector (Ad) into cells, was synthesized. The synthetic peptide was conjugated to Ad, which potentially can act as an efficient carrier of heterologous genes into cells. The Tat-related peptide was synthesized using the solid phase method and then was coupled to the heterofunctional cross-linking reagent, 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester. The resulting peptide-succinimidohexanoic acid *N*-hydroxysuccinimide ester was conjugated to Ad containing the luciferase gene. B16BL6 cells infected with the peptide-conjugated Ad luciferase gene construct exhibit a 50-fold greater luciferase activity than B16BL6 cells infected with wild-type Ad containing the luciferase gene.

Gene therapy has attracted much attention as a potential clinical treatment/cure for intractable diseases.[1] A key to the successful implementation of gene therapy protocols is the design of the transgenesis vector. Adenovirus vectors (Ad) are often used as transport agents during gene therapy experiments and trials since they exhibit suitable transduction and gene-expression properties; but for routine clinical procedures, more efficient transfer vectors need to be developed. Previously, we showed that an Arg-Gly-Asp(RGD)-related peptide that binds to integrin functions as an efficient auxiliary transporter of Ad.[2] The RGD-related peptide, when covalently bound to Ad, transports Ad into dendritic cells via interaction with integrins. For this report, a different type of Ad auxiliary transporter was designed, synthesized, and shown to greatly increase the amount of Ad (containing the luciferase gene) transferred into cells.

The human immunodeficiency virus (HIV)-1 protein, Tat, is a transcriptional activator of HIV and can cross both the plasma and nuclear membranes. Tat contains 86 amino acids, but its translocation activity is associated with the peptide sequence, Tat(48–60), (GlyArgLysLysArgArgGlnArgArgArgProProGln: GRKKRRQRRRPPQ).[3] Futaki et al. reported that certain synthetic arginine-rich peptides can readily cross cell membranes and that the optimal number of arginines required for efficient translocation is approximately eight.[4] We designed the peptide, acetyl-Gly-Arg-Arg-Arg-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Gly-Cys amide (Ac-GRRRRRQRRRPPQGC-NH_2), to be an efficient auxiliary transporter of Ad. Since Futaki et al.[4] reported that the number of arginine residues correlates with translocation ability, the sequence, Ac-GRRRRRQRRRPPQGC-NH_2, was designed so that the two lysines found in Tat(48–60) were replaced with arginines. A C-terminal cysteine was added so that the peptide could be linked to Ad through the heterofunctional cross-linking reagent 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (MHS),[5] which reacts with amine and sulfhydryl moieties (Fig. 1). The peptide was synthesized using an Applied Biosystems Peptide Synthesizer 433A-1. 9-Fluorenylmethoxycarbonyl (Fmoc) amino acids [Fmoc-Gly-OH; Fmoc-Pro-OH; N^{α}-Fmoc-N^{G}-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfony-

Keywords: Adenovirus vector; Tat; Tat-peptide; Peptide synthesis.
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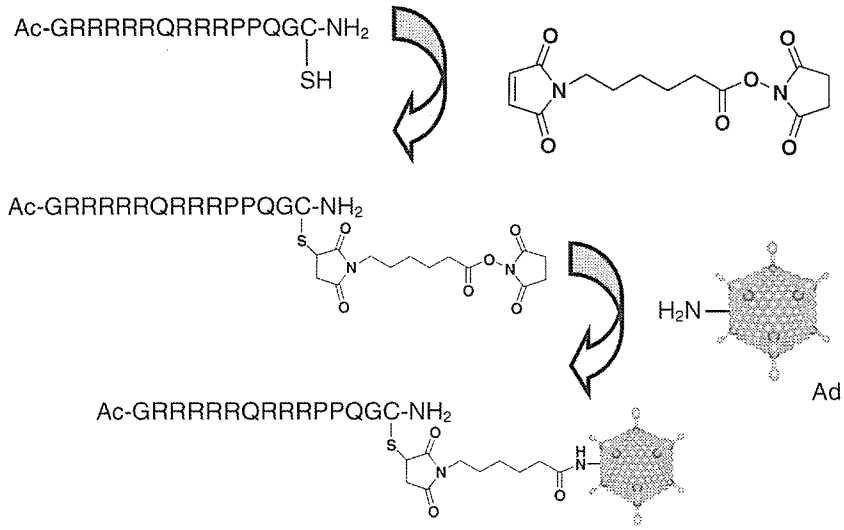

Figure 1. Synthesis of a peptide–Ad conjugate that acts as an efficient heterologous gene transporter.

larginine: Fmoc-Arg(Pbf)-OH; N^{α}-Fmoc-*S*-tritylcysteine: Fmoc-Cys(Trt)-OH; and Fmoc-Gln(Trt)-OH] were coupled in a stepwise manner to Rink amide resin[6] (PE Biosystems. Amino content: 0.67 mequiv/g, 379 mg, 0.25 mmol) using the coupling reagent, 2-(1-*H*-benzotriazole-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU),[7] in *N*-methylpyrrolidone (NMP). After each coupling step, the Fmoc group was removed using 20% piperidine/NMP. The synthetic Fmoc-Gly-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Gln(Trt)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Pro-Pro-Gln(Trt)-Gly-Cys(Trt)-Rink amide resin was treated with 20% piperidine/NMP and then treated with acetic anhydride. The peptide was cleaved from the resin with trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (95:2.5:2.5). The resulting crude peptide, (Ac-GRR RRRQRRRPPQGC-NH_2, 410 mg yield), was purified using RP-HPLC.[8] The HPLC profile of the crude peptide mixture is shown in Figure 2 The yield of the purified peptide was 202 mg (28% as calculated from the amino content of the used resin).

The purified peptide (40 mg, 14 μmol), dissolved in PBS (pH 7.2, 500 μL), and the heterofunctional cross-linkage reagent (MHS: 4.3 mg, 14 μmol), dissolved in dimethylsulfoxide (DMSO, 10 μL), were combined and then stirred for 0.5 h. We attempted to purify the product, Ac-GRRRRRQRRRPPQGC(SHS)-NH_2 (SHS: 6-succinimidohexanoic acid *N*-hydroxysuccinimide ester), using HPLC, but could not—the *N*-hydroxysuccinimide ester hydrolyzes easily in water; therefore, the reaction product mixture was frozen immediately and kept at −80 °C until needed. While gently stirring, Ad-Luc, whose chromosome encodes the heterologous luciferase gene, was reacted with Ac-GRRRRRQRRRPPQGC(SHS)-NH_2 at 37 °C for 45 min. To test the relative transduction efficiency of the peptide–Ad conjugate [Tatpep-(Ad-Luc)],

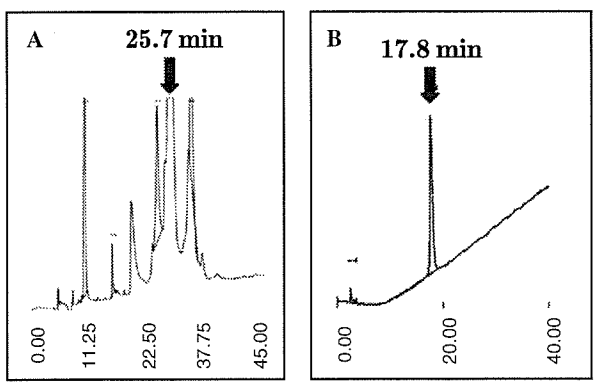

Figure 2. HPLC profile of synthetic crude Ac-GRRRRRQ-RRRPPQGC-NH_2. (A) Preparative HPLC of crude synthetic peptide. Column: DAISOPAK SP-120-5-ODS-B (20 × 250 mm). Flow rate: 10 mL/min. Eluent: CH_3CN/H_2O containing 0.05% CF_3COOH. Gradient: 10–70% CH_3CN over the course of 60 min. The absorbance was measured at 220 nm. (B) Analytical HPLC of purified sample. Column: Inertsil ODS-3 (4.6 × 250 mm). Flow rate: 1 mL/min. Eluent: CH_3CN/H_2O containing 0.05% CF_3COOH. Gradient: 5–20% CH_3CN over the course of 40 min. The absorbance was measured at 220 nm.

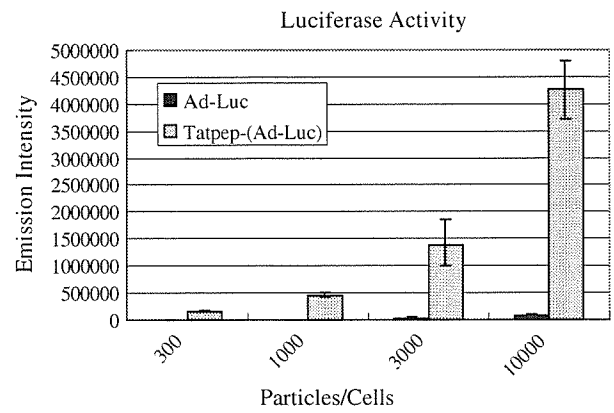

Figure 3. Transduction efficiency of Ad-Luc and Tatpep-(Ad-Luc) into B16BL6 cells Cells (2×10^4) were incubated with 300, 1000, 3000 or 10,000 particles/cell of Ad-Luc (solid bars) or Tatpep-(Ad-Luc) (gray bars). Luciferase activity, which was determined using a Luciferase Assay System Kit (Promega, USA) and a Microlumat Plus LB96 (Perkin-Elmer, USA) after lysing the cells with Luciferase Cell Culture Lysis Reagent (Promega, USA), was measured at the end of a 24 h incubation. The bars report the mean relative unit of light per well ± SD ($n = 3$).

B16BL6 cells were incubated with it or with Ad-Luc for 24 h, at which time luciferase activity was measured (Fig. 3). B16BL6 cells were used because the Coxackie-adenovirus receptor, which transports Ad across the plasma membrane, is nearly absent.[9] At concentrations of 300 and 1000 particles/cell, cells that were exposed to Ad-Luc did not glow, while those exposed to Tatpep-(Ad-Luc) construct clearly did. Ad-Luc infected cells glowed weakly at doses of 3000 and 10,000 particles/cell, while Tatpep-(Ad-Luc) exhibited strong luciferase activity at the same concentrations. The transduction activity of Tatpep-(Ad-Luc) is about 50-fold greater than that of Ad-Luc—a remarkable finding.

In summary, we designed the peptide, Ac-GRRRRRQ-RRRPPQGC-NH_2, to be an efficient auxiliary transporter of Ad into cells. Ad, when covalently bound to this synthetic peptide, exhibits a transduction ability 50-fold greater than does Ad alone. This modified Ad is a promising experimental tool for transduction studies.

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reviews

Adenovirus Vector-Mediated Gene Transfer into Stem Cells

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Abstract: Stem cells, including embryonic stem (ES) cells, mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs), are defined by their capacity for self-renewal and multilineage differentiation. Efficient gene transfer into stem cells is essential for the basic research in developmental biology and for therapeutic applications in gene-modified regenerative medicine. Adenovirus (Ad) vectors, based on Ad type 5, can efficiently and transiently introduce the exogenous gene into many cell types via the primary receptor, coxsackievirus, and adenovirus receptor (CAR). However, some kinds of stem cells, such as MSCs and HSCs, cannot be efficiently transduced with conventional Ad vectors based on Ad serotype 5 (Ad5), because of the lack of CAR expression. To overcome this problem, fiber-modified Ad vectors and an Ad vector based on another serotype of Ad have been developed. Here, we review the advances in the development of Ad vectors suitable for stem cells and discuss their application in basic biology and clinical medicine.

Keywords: Adenovirus; stem cell; gene therapy; regenerative medicine; review

Introduction

Adenovirus (Ad) is a nonenveloped virus containing an icosahedral protein capsid with a diameter of approximately 80 nm. At least 51 serotypes of human Ad have been identified and classified into six different subgroups (A–F), many of which are associated with respiratory, gastrointestinal, or ocular diseases. Of them, Ad serotype 5 (Ad5) and Ad serotype 2, both belonging to subgroup C, have been the most extensively studied for use as vectors in gene therapy applications. Ad capsids consist of three major protein components: the hexon, the penton base, and the fiber. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices (Figure 1A). The two components of the penton capsomer, the fiber and penton base, interact with distinct cell surface receptors during the entry of Ad into susceptible cells. Fiber proteins consist of three distinct domains: the tail, the shaft, and the knob. Each domain has distinct functions in host cell infection. The amino-terminal tail anchors the fiber to the Ad capsid through association with the penton base.[1] The shaft extends away from the virion surface and, in Ad5, is composed of 22 pseudorepeats of 15 amino acids in a triple-β-spiral conformation.[2] By extending the knob away from the virion, the shaft facilitates its interaction with the host

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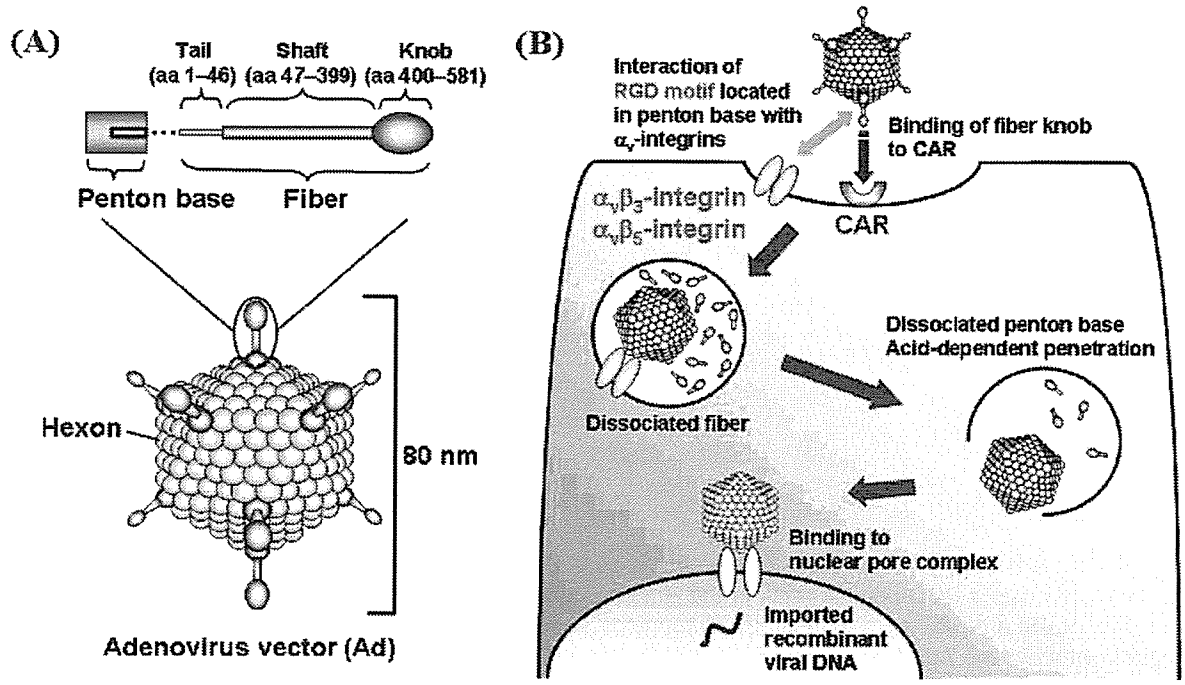

Figure 1. Structure and gene transduction pathway of the Ad vector. (A) The double-stranded virus genome is packaged within an icosahedral protein capsid. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices. The fiber is composed of the tail, shaft, and knob domain. (B) The Ad vector binds to CAR following internalization in the cells and releases the viral DNA into the nuclei.

receptor.[1] The trimeric subunits of the carboxyl C-terminal knob domain are responsible for binding to the host's primary cellular receptor.[3,4]

Human Ad5 contains a linear, approximately 36 kb, double-stranded DNA genome encoding more than 70 gene products. The viral genome contains five early transcription units (E1A, E1B, E2, E3, and E4), two early delayed (intermediate) transcription units (pIX and IVa2), and five late units (L1–L5), which mostly encode structural proteins for the capsid and internal core. Inverted terminal repeats (ITRs) at the end of the viral genome function as replication origins. The E1A gene is the first transcription unit to be activated shortly after infection and is essential to the activation of other promoters and the replication of the viral genome. In the first-generation Ad vectors, the E1 (E1A and E1B) gene is deleted and the virus propagated in E1-transcomplementing cell lines, such as 293,[5] 911,[6] or PER.C6 cells.[7] The E3 region-encoded proteins modulate the host defense but are not required for viral replication in vitro; thus, the E3 region is often deleted to enlarge the packagable size limit for foreign genes. Since up to 3.2 and 3.1 kb of the E1 and E3 regions, respectively, can be deleted[8] and approximately 105% of the wild-type genome can be packaged into the virus without affecting the viral growth rate and titer,[9] E1/E3-deleted Ad vectors allow the packaging of approximately 8.1–8.2 kb of foreign genes.[8]

The coxsackievirus and adenovirus receptor (CAR), which is a broadly distributed type I membrane protein, has been identified as the primary receptor for Ad of subgroups A and C–F.[10–12] The entry of Ad5 into cells is initiated by the

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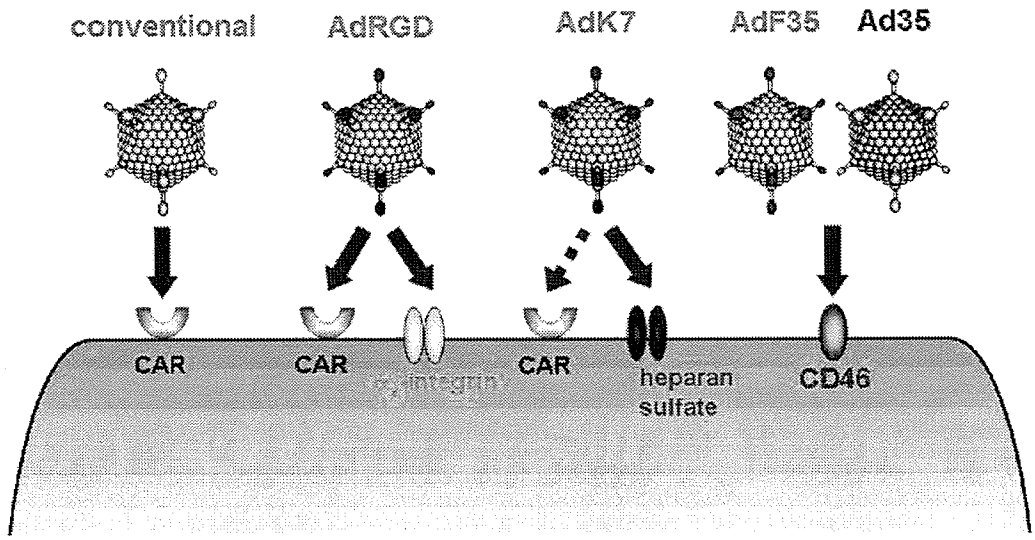

Figure 2. Characteristics of gene delivery by various types of Ad vectors. The conventional Ad vector infects via CAR. The AdRGD vector contains a RGD peptide motif in the HI loop of the fiber knob and infects via αv integrin as well as CAR. The AdK7 vector contains a polylysine peptide in the C-terminus of the fiber knob and infects via heparan sulfate as well as CAR. It is uncertain whether the AdK7 vector infects via CAR. The Ad35 and AdF35 vectors, which contain a fiber protein derived from the Ad5 fiber tail and the Ad35 fiber knob and shaft, infect via CD46.

attachment of fiber on the surface of the capsid to the CAR on the cell surface (Figure 2). The affinity of the RGD (Arg-Gly-Asp) peptide at the penton base of the Ad5 capsid for the cell surface molecules of the integrin family, such as $\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_1$, aids in the internalization of Ad5 into the cell.[13−15] Furthermore, heparan sulfate glycosaminoglycans have also been reported to serve as primary attachment sites for Ad2 and Ad5.[16] The abundant expression of these receptors in various cells determines the wide tropism of Ad vectors. Internalized Ad reaches the endosomal pathway and avoids lysosomal degradation (Figure 1B). Inside the endosome, a stepwise disassembly program takes place, allowing the Ad to release its genome into the nucleus. During this process, the pH of the endosome decreases, leading to the release of the fiber from the virion and the dissociation of the penton base.[17] The resulting endosome rupture allows viral DNA to escape from inside the degraded capsid and to enter the nucleus (Figure 1B). During this process, the terminal protein plays a crucial role in translocating the Ad genome into the nucleus. This uncoating process of the Ad starts immediately after internalization and ends 40 min after infection with the translocation of the Ad into the nucleus. As early as 60 min after infection, the Ad begins to transcribe its genome in the host cell.[18]

Although Ad vectors mediate extremely high transduction efficiency, gene transfer with Ad vectors is less efficient in some kinds of cells, such as mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), dendritic cells, T cells, smooth muscle cells, skeletal muscle cells, and others because of the scarcity of CAR on their cell surfaces. Modification of the Ad fiber proteins has been used to successfully overcome this obstacle.[19,20] One is constructed by the addition of foreign peptides to the HI loop or C-terminus of the fiber knob of an Ad vector.[21−25] Enhanced gene transfer has been

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reported, on the basis of the use of mutant fiber proteins containing either an RGD peptide (AdRGD vector)[21−26] or a stretch of lysine residues [K7 (KKKKKKK) peptide] (AdK7 vector),[21,25,26] which target αv integrins or heparin sulfates on the cell surface, respectively (Figure 2). Altered vector tropism was reported with the substitution of the Ad5 fiber protein with that of Ad belonging to subgroup B, such as Ad types 3, 11, and 35.[27−31] These fiber-modified Ad vectors infect cells via CD46, CD80, and CD86, which have recently been identified as the cellular receptors of Ad belonging to subgroup B (Figure 2).[32−36] Mercier et al. described the creation of a chimeric Ad vector encoding the reovirus attachment protein σ1, which targets cells expressing junctional adhesion molecule 1.[37]

Several groups have developed an Ad vector from the entire Ad type 35 (Ad35) or Ad type 11 (Ad11) and have demonstrated that the Ad35 and Ad11 vectors exhibit higher transduction efficiencies into hematopoietic progenitor and dendritic cells compared with the conventional Ad5 vector (Figure 2).[38−43] As other approaches to changing the vector tropism, modification of the Ad vector with the antibodies, the fusion protein composed of CAR and the cell binding domain, cationic lipid, or macromolecules has been reported.[19,20] Here, we highlight the genetic manipulations of stem cells by the Ad vector and fiber-modified Ad vector for basic research and therapeutic usage. Recent advances in Ad vector-mediated gene transfer into stem cells, such as embryonic stem (ES) cells, mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs), will be discussed.

Gene Transfer into Stem Cells

Stem cells are defined as cells which possess the abilities of self-renewal and multilineage differentiation. Stem cells have been isolated from a wide variety of tissues, and in general, their differentiation potential may reflect the local environment. They lack tissue-specific characteristics but under the influence of appropriate signals can differentiate into specialized cells with a phenotype distinct from that of their precursor. Gene therapy applications that target stem

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cells offer great potential for the treatment of many kinds of diseases. Despite this promise, clinical success has been limited by poor rates of gene transfer and poor levels of gene expression. Therefore, an efficient gene delivery system needs to be developed for stem cell gene therapy.

Gene Transfer into Embryonic Stem Cells. ES cells are pluripotent cell lines derived from the inner cell mass of the developing blastocyst.[44−46] With the establishment of human ES (hES) cells, they have been used as a renewable source of transplantable tissue-specific stem cells.[47−49] ES cells differentiate spontaneously in vitro in a random manner into a mixture of differentiated cells. The protocols for the differentiation of ES cells enriched for a specific lineage have been developed in both the mouse ES (mES)[50,51] cell and hES cell systems,[52,53] although the differentiated cells are still relatively heterogeneous. Therefore, further research is needed to allow controlled directed differentiation of ES cells into pure cultures of committed cells. One of the most powerful techniques for controlled differentiation is genetic manipulation. Electroporation methods,[54] retroviral vectors,[55,56] lentiviral vectors,[57−59] and a supertransfection method based on a replication system using the polyoma replication origin and large T antigen[60] have been used for exogenous gene expression in ES cells, although lentiviral vectors have been shown to be ineffective at expressing exogenous genes in mES cells, but not in hES cells.[57,59] In plasmid-based systems such as eletroporation and supertransfection methods, stable cell lines are generated by selection using a drug resistance gene. All these methods mediate long-term constitutive gene expression, although a long-term gene expression system such as that as described above may be problematic for use in therapeutic applications, because the gene is continuously expressed even after cell differentiation. There is thus a need for efficient vector systems for transient expression.

The Ad vector has been thought to be inappropriate for gene transfer into ES cells.[61] It has been reported that the retrovirus vector preferentially transduced ES cells, while the Ad vector containing the cytomegalovirus (CMV) promoter preferentially transduced embryonic fibroblasts as feeders in the ES culture.[61] However, it was found that the

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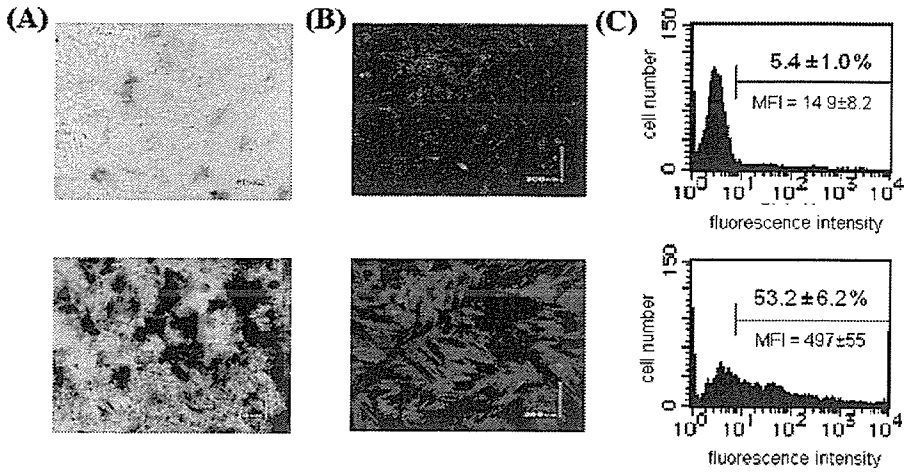

Figure 3. Improved transduction efficiency in the stem cells by the optimized Ad vectors. (A) mES cells were transduced with the LacZ-expressing conventional Ad5 vector containing the CMV promoter (top) or EF-1α promoter (bottom). (B) hMSCs were transduced with the LacZ-expressing Ad5 vector (top) or AdK7 vector (bottom). Both vectors have the CA promoter. (C) Human CD34+ cells were transduced with the GFP-expressing Ad5 vector (top) or Ad35 vector (bottom). Both vectors have the CMV promoter. MFI is the mean fluorescence intensity.

choice of a promoter is important for the efficient expression of exogenous genes in mES cells (Figure 3A). In the transient expression system using a cationic liposome−plasmid complex, the EF-1α (elongation factor 1α) and CA promoter (β-actin promoter/CMV enhancer) were shown to be highly active in mES cells while the CMV promoter was inactive.[62] More recently, we reported that the Ad vector containing the EF-1α or CA promoter has mediated the efficient expression of the reporter gene in mES cells, whereas the Ad vector containing the Rous sarcoma virus (RSV) or the CMV promoter has exhibited little expression.[63] Because CAR was highly expressed in mES cells but not in feeder cells,[63] the Ad vector could be a powerful tool for the genetic manipulation of mES cells when an appropriate promoter is used. To date, although we have no idea about the expression of CAR in hES cells, the Ad vector was reported to mediate the reporter gene expression in both mES cells and hES cells,[64] suggesting that hES cells may also express CAR on their cell surfaces.

As a result of the comparative analysis of mES cells transduced with various types of fiber-modified Ad vectors, the conventional Ad vector exhibited highly efficient and specific transduction, whereas the AdRGD and AdK7 vectors transduced mES cells and feeder cells (embryonic fibroblasts) to the same degree.[63] Therefore, the conventional Ad vector containing the EF-1α or CA promoter should be appropriate when only ES cells are transduced. In turn, the AdRGD or AdK7 vector is adequate when both ES cells and feeder cells are transduced.

The conventional Ad vector containing the EF-1α promoter was applied for the transduction of functional genes. It is well-known that the activation of signal transducer and activator of transcription 3 (STAT3) is essential for leukemia inhibitory factor (LIF)-mediated mES cell self-renewal, and the inhibition of LIF/STAT3 signaling leads to either apoptosis or differentiation.[65] It is also known that transcription factor Nanog maintains the pluripotency of mES cells in a manner that is independent of LIF/STAT3 signaling.[66,67] Ad vector-mediated STAT3F (STAT3 dominant-negative mutant) transduction strongly promoted mES cells to cell differentiation into three germ layers without any nonspecific toxicity.[63] The co-infection of the STAT3F-expressing Ad vector and the Nanog-expressing Ad vector showed that the differentiation suppressing ability of Nanog negated the differentiation promoting function of STAT3F and that mES cells maintained their undifferentiated state.[63] Thus, the differentiation of ES cells could be controlled by the transduction of differentiation-key regulator genes with the Ad vector. ES cells might differentiate into hematopoietic progenitor, pancreatic β cells, or neurons by the Ad vector-mediated introduction of HoxB4,[68,69] Pax4,[70] or nuclear receptor-related I,[71] respectively.

Gene Transfer into Mesenchymal Stem Cells. MSCs, which reside within the stromal compartment of bone

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marrow, were first identified as bone-forming progenitor cells from rat marrow.[72] MSCs represent a very small fraction, 0.001−0.01% of the total population of nucleated cells in marrow.[73] They have the capacity to differentiate into cells of connective tissue lineages, including bone, fat, cartilage, and muscle. Recently, it has been reported that MSCs can differentiate into other lineages, such as neurons,[74] hepatocytes,[75] and insulin-producing cells.[76] Therefore, MSCs have attracted a great deal of interest because of their potential use in regenerative medicine and tissue engineering. To date, MSCs could be differentiated in vitro into proper lineages via a change in the culture conditions.[77] Another method for the in vitro differentiation is to genetically modify MSCs.[78,79] Although exogenous gene transfer into human MSCs (hMSCs) has been reported by using a conventional Ad vector, its transduction efficiency is quite low due to the scarcity of CAR.[80,81] Therefore, hMSCs have been transduced with high titers (more than 1000 infectious units/cell) of Ad vectors.[80,81] Fiber-modified Ad vectors have been applied for hMSCs to improve the transduction efficiency.[79,82,83] hMSCs infected with the AdRGD vector containing the BMP2 gene produced larger amounts of BMP2 than cells infected with the conventional Ad vector and efficiently differentiated into the osteogenic lineage.[82,83] Highly efficient transduction of hMSCs was achieved with tropism-modified Ad5 vectors carrying fiber shaft domains and knobs of different serotypes of Ad, such as Ad16, Ad35, or Ad50.[84] In a systematic comparison with various types of fiber-modified Ad vectors, the AdK7 vector is the most efficient for hMSCs and exhibited a 460-fold higher transduction efficiency than the conventional Ad vector.[79] The AdRGD vector or the Ad vector containing the Ad35 fiber (AdF35) exhibits a 16 or 130 times higher transduction efficiency, respectively, than the conventional Ad vector.[79] hMSCs are found to express CD46, which is the primary receptor for Ad35, but not CAR.[79] In conclusion, the AdK7 or AdF35 vector is the most appropriate for the transduction of hMSCs (Figure 3B).

Gene Transfer into Hematopoietic Stem Cells. Hematopoietic stem cells (HSCs) are capable of self-renewal and multilineage differentiation into all mature blood cells.[85] HSCs comprise only 0.01% of the whole bone marrow, the tissue in which they primarily reside.[86] Efficient transduction into HSCs would afford the opportunity to treat a number of hematopoietic disorders and would be a powerful tool for

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the study of the proliferation, differentiation, and trafficking of HSCs. Although the retroviral and lentiviral transduction of HSCs to achieve stable gene expression has been established,[87,88] stable expression is not always desirable. For example, stable expression of MDR1 gene results in HSC expansion but can cause leukemia upon transplantation to recipient mice.[89] As the Ad vector mediates the exogenous gene expression transiently, this vehicle can be safe for gene therapy. However, the application of conventional Ad vectors for the transduction into human CD34+ cells, which contain a population of HSCs, has been limited because CAR is not expressed at sufficient levels in human CD34+ cells.[90,91] It has been shown that Ad serotype 35 (Ad35), which belongs to subgroup B, is efficient at binding to human CD34+ cells and hematopoietic cell lines.[90,92] We showed that the Ad35 vector, which is composed from the whole Ad35, achieved higher levels of transduction efficiency in human bone marrow CD34+ cells than both conventional Ad5 vectors and AdF35 vectors.[39,93] The expression level of reporter genes in the CD34+ cells transduced with the Ad35 vector was 12−76 and 1.4−3 times higher than that in the cells transduced with the Ad5 and AdF35 vectors, respectively.[39] The transduction efficiency of the Ad35 vector was slightly higher than that of the AdF35 vector, although the reason remains unknown. CD46 is ubiquitously expressed in almost all human cells, including human cord blood CD34+ cells.[94] Therefore, human CD34+ cells would be considered to be a suitable target for the Ad35 vector (Figure 3C). As a result of the systematic comparison of promoters with Ad35 vectors, significantly higher transduction efficiencies were achieved with the EF-1α, CA, and CMV promoter/enhancer with the largest intron of CMV (intron A) (CMVi) promoters. In particular, the CA promoter was found to allow for the highest transduction efficiencies in both the whole human CD34+ cells and the immature subsets.[93] In mice, a population of mouse bone marrow highly enriched for HSC, called side population (SP) cells, has been reported to be transduced with the conventional Ad5 vector.[95] This suggests that pure mouse HSCs might express CAR on the cell surface. Further studies are needed to clarify this. The Ad vector-mediated transduction of hematopoietic regulator genes, such as HoxB4,[68,69] Bmi-1,[96] or SCL/Tal-1,[97] into HSCs may be effective for therapeutic use such as HSC expansion, although the Ad vector expressing HoxB4 was unsuccessful because of unexpected HSC differentiation due to its high transduction efficiency.[98]

Conclusions

We have reviewed recent advances in the development of improved Ad vectors for stem cells. Ad vectors have advantages over other viral vectors: the high transduction efficiency, the ease of vector preparation, and the transient expression ability. By the Ad vector-mediated introduction of a differentiation master regulator gene, we could control the differentiation of stem cells. These technical advances should greatly facilitate the analysis of gene function in the stem cells as well as the therapeutic applications of gene-modified stem cells.

Abbreviations Used

ES, embryonic stem; mES, mouse ES; MSCs, mesenchymal stem cells; HSCs, hematopoietic stem cells; Ad, adenovirus; CAR, coxsackievirus and adenovirus receptor; Ad5, Ad serotype 5; ITR, inverted terminal repeats; Ad35, Ad serotype 35; AdRGD vector, Ad vector containing the RGD

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peptide; Ad K7 vector, Ad vector containing a polylysine stretch; hES, human ES; STAT3, signal transducer and activator of transcription 3; LIF, leukemia inhibitory factor; STAT3F, dominant-negative mutant of STAT3; hMSCs, human MSCs; BMP2, bone morphogenetic protein 2; AdF35, Ad vector containing the Ad35 fiber.

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アデノウイルスベクター開発の最前線

水口裕之

従来のアデノウイルスベクターの問題点（免疫反応を生じることや感染域の制限など）を克服し，機能性（ターゲティング能の付与など）に優れた次世代アデノウイルスベクターの開発が進んでいる．本稿では，主に遺伝子工学的手法を用いたアデノウイルスベクターの改良研究の最前線について解説する．

はじめに

遺伝子治療の実用化といっそうの進展に向けての最大の鍵は，高い安全性を確保し，治療用目的遺伝子を必要な細胞に効率良く導入し，目的に応じて自由に発現させる技術の開発である．これまで施行されてきた遺伝子治療臨床研究は，主に1990年代に開発された古典的なベクターを用いたものであり，米国でのアデノウイルスベクター投与に伴う死亡事故や，フランスでの白血病の発症といった例外的な事例はあるものの，安全面での評価はほぼ終わり，今後はより有効性に重点を置いた臨床研究が主流になってくるものと思われる．そのためには，機能面で優れた改良型ベクターの開発と実用化が必要不可欠であり，これにより遺伝子治療の安全性・有効性の大幅な増進が期待できる．

アデノウイルスベクターは，①既存のベクターでは最も遺伝子導入効率が良いこと*1，②導入遺伝子が宿主染色体へ組み込まれることなく，染色体外にエピソームとして存在することから，一過性の遺伝子発現を示すこと*2，③他のウイルスベクターに比べ圧倒的に高いタイター（力価）のベクター（通常他のベクターに比べ1,000倍以上）が得られること，などの長所を有し，ベクターとしての優れた基本的性質を有している．

一方，①遺伝子導入が標的細胞のアデノウイルス受容体（coxsackievirus and adenovirus receptor：CAR）の発現レベルに依存し，CARを発現していない細胞への適用が困難なこと，②組織特異性を示さないこと，③免疫反応を伴うこと，などの問題点を有し，これらの問題を克服し，機能面で優れた次世代アデノウイルスベクターの開発がわれわれや欧米を中心に盛んに行われている．本稿では，次世代アデノウイルスベクター開発の最前線について解説する．

1．標的細胞指向性の制御

i）遺伝子導入時のCAR依存性を克服したアデノウイルスベクターの開発

従来用いられているアデノウイルスベクターは，サブグループCに属する5型（あるいは2型）のヒトアデノウイルスを基盤としている．ヒトアデノウイルスはAからFまでのサブグループに分けられ，少なくとも51種類のserotypeが知られているが，サブグループBに属するウイルスを除き，多くのアデノウイルスは受容体としてCARを認識して細胞に感染する（図1）[1]．CARの発現が乏しいために従来の5型アデノウイルスベクターでの効率の良い遺伝子導入が困難な細胞種は意外と多く，造血幹細胞をはじめとする血液系細胞，樹状細胞，間葉系幹細胞，血管平滑筋細胞，骨格筋細胞，滑膜細胞などが知られている．また，癌細胞は悪性度の進行とともに，CARの発現低下，およびアデノウイルスベクターでの遺伝子導入効率の低下が報告されており[2]，本ベクターを用いて癌を対象とした遺伝子治療臨床研究を進めるうえで考慮すべき問題と考えられている．

＊1：センダイウイルスベクターも遺伝子発現効率に優れていることが報告されているが，本ベクターは他の一般的なウイルスベクターと異なり，細胞内に導入された遺伝子が増幅するという特異な性質を有することから，他のベクターと同列に比較することは困難である．

＊2：細胞増殖に伴い導入遺伝子が希釈されるため，一過性の遺伝子発現を示す．一方，分化した増殖停止期の細胞に対しては，後述するようにアデノウイルスに対する免疫の問題が克服されれば，数ヶ月以上の長期間の遺伝子発現を示すことが知られている．

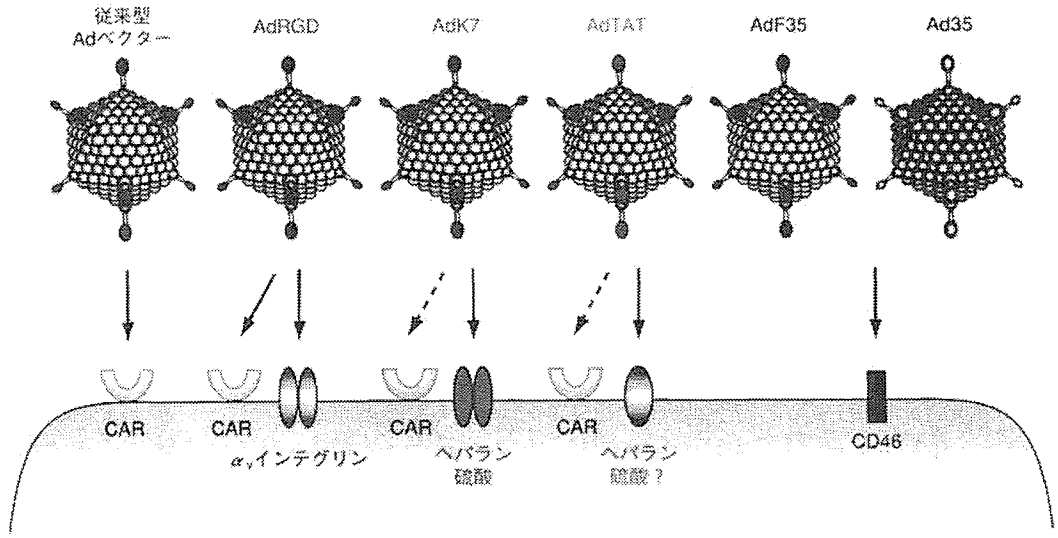

図1 ●各種改良型アデノウイルスベクター

野生型のファイバーをもった従来型の5型アデノウイルスベクターは細胞表面上の受容体であるCARを認識して感染するが，RGD配列やポリリジン配列をファイバーに有したファイバー改変ベクター（AdRGD，AdK7）はCARだけでなくαvインテグリンやヘパラン硫酸を認識しても感染できる．TATペプチドを付与したAdTATは，詳細な細胞内移行メカニズムは不明であるが，CAR非依存的に感染できる．また，35型アデノウイルスのファイバーを有したベクター（AdF35）や，すべての構造タンパク質が35型アデノウイルスからなるベクター（Ad35）は，CD46を認識して感染する

アデノウイルスベクターによる遺伝子導入時のCAR依存性を克服するために，ファイバータンパク質[*3]を改変した改良型ベクターの開発が進んでいる．例えば，αvインテグリンに親和性があるRGD（Arg-Gly-Asp）ペプチドや，ヘパラン硫酸に親和性があるポリリジンペプチドをファイバー表面上に遺伝子工学的に表現させることにより，CARを発現していない細胞に対しても効率良く遺伝子導入できる（図1）[3,4]．われわれは最近，HIV（human immunodeficiency virus）由来のタンパク質導入ドメイン（Protein Transduction Domain：PTD）として知られているTatペプチドをファイバーに付与することで，RGD配列やポリリジン配列を付与したベクターよりも，より広範に効率良く外来遺伝子を発現可能であることを見出し，その応用が期待される．

また，ファイバー部位をCAR以外の分子を認識する他の血清型のアデノウイルスのファイバーに置換することでも，遺伝子導入時のCAR依存性を克服することができる．例えば，ヒト由来細胞であればほとんどすべての細胞に発現が認められるCD46を受容体としている11・35型アデノウイルス由来のファイバーを付与することで，5型アデノウイルスベクターでの効率の良い遺伝子導入が困難な造血幹細胞，樹状細胞などへの効率の良い遺伝子導入が可能になる[5,6]．

ii）ターゲティング能を有した
アデノウイルスベクターの開発

ターゲティング能を有したベクターの開発は，全身投与での治療効果が期待できるだけでなく，たとえ局所にベクターを投与した場合においても，標的細胞以外への感染，拡散を防ぐことが期待できることから，重要な研究課題である．目的の組織でだけ遺伝子を発現させることが可能なアデノウイルスベクターの開発には，①キャプシドタンパク質の遺伝子工学的な改変，②抗体やタンパク質，高分子を用いてのベクター表面の修飾，あるいは③組織特異的プロモーターの利用などの方法がある．最終的には，これらの組み合わせが好ましいが，ベクター自身を

＊3：ファイバータンパク質
テール，シャフト，ノブからなり，ノブ領域がCARと結合する．