of PBS was administered into the jejunal loop. Blood was collected from the jugular vein at the indicated time points. The plasma concentration of FD-4 was determined with a fluorescence spectrophotometer (Fluoroskan Ascent FL; Thermo Electron Corp., Waltham, MA). The area under the plasma concentration–time curve from 0 to 4 h (AUC_{0-4}) was calculated by the trapezoidal method.

3. Results

3.1. *Roles of Tyr306, Tyr310 and Tyr312 in the interaction between C-CPE and claudin-4*

We previously found that the C-terminal 16 amino acids are responsible for ability of C-CPE to modulate the TJ barrier and to bind to claudin-4 [22,23] (Fig. 1). Kokai-Kun et al. showed that substitution of Tyr310 with Cys reduced binding of CPE to the brush border membrane in rabbits [24]. Here, we focused on three tyrosine residues in this region, namely Tyr306, Tyr310 and Tyr312. To evaluate the function of these tyrosine residues, we generated Tyr306, Y310A and Y312A mutants by site-directed mutagenesis. We then examined the ability of these mutants to inhibit the toxicity of C-CPE-PSIF, a molecule that specifically targets and is toxic to claudin-4-expressing cells [25]. In CL4/L cells, pretreatment with C-CPE attenuated toxicity of C-CPE-PSIF in a dose-dependent manner (Fig. 2A). The Y306A, Y310A and Y312A mutants had reduced abilities to inhibit C-CPE-PSIF-induced cytotoxicity. We also used a pull-down assay to examine the ability of these mutants to interact with claudin-4 in Caco-2 lysates, which have well-developed TJs [22]. Less claudin-4 precipitated with the Y306A and Y310A mutant than with C-CPE, but the Y312A mutant bound claudin-4 as effectively as C-CPE (Fig. 2B). The extra band below claudin-4 was observed in C-CPE, mutants and C-CPE/mutants-treated samples. The extra band was due to non-specific reaction of anti-claudin-4 Ab with histidine-tag (data not shown).

3.2. *Interaction of double mutants of C-CPE with claudin-4*

To evaluate the synergistic effects of Tyr306, Tyr310, and Tyr312 on the ability of C-CPE to interact with claudin-4, we generated double tyrosine to alanine substitution mutants (Y306A/Y310A, Y306A/Y312A, and Y310A/Y312A). As shown in Fig. 3A, we investigated the interaction of double mutants with claudin-4 in the C-CPE-PSIF competitive assay. Pretreatment of cells with C-CPE at 10 μg/ml inhibited LDH release to 18% of the vehicle-treated group, whereas treatment of cells with the Y306A/Y310A and Y306A/Y312A mutants did not affect C-CPE-PSIF-induced LDH release even at 10 μg/ml. Treatment of cells with Y310A/Y312A at 10 μg/ml partially attenuated the cytotoxicity of C-CPE-PSIF. A pull-down assay revealed that the Y306A/Y310A and Y306A/Y312A mutants completely lost the ability to bind claudin-4. Y310A/Y312A mutant had the partly reduced ability to bind claudin-4 (Fig. 3B). Thus, mutation of Tyr310 and Tyr312 to alanine reduced precipitation of claudin-4 in the pull down assay.

3.3. *Effects of double mutants on TJ barrier function in Caco-2 cells*

Next, we investigated the effects of the mutants on the TJ barrier function in Caco-2 monolayers grown in Transwells. Treatment of the cells with C-CPE, the Y310A and Y312A mutant for 18 h reduced the TER value, a marker of tightness in the TJs, from 295 to 30 and from 326 to 49 Ω cm^2, respectively (Fig. 4A). The Y306A and Y310A/Y312A mutants caused less of a reduction in TJ barrier function than C-CPE. The Y306A/Y310A and Y306A/Y312A mutants, however, had almost no effect on the TJ barrier function (from 291 to 261 and from 289 to 254 Ω cm^2, respectively; Fig. 4A).

We previously found that C-CPE enhances rat jejunal absorption of FD-4 by interacting with claudin-4 [23]. We further evaluated the ability of each mutant to enhance jejunal absorption of FD-4 (Fig. 4B and C). We found that the Y310A mutant enhanced absorption to a similar extent as C-CPE,

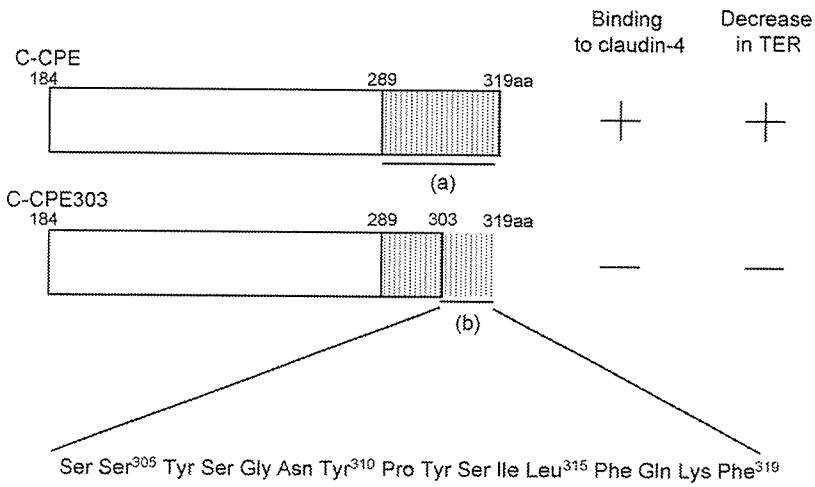

Fig. 1 – Diagram of C-CPE structure. C-CPE is the C-terminal fragment of CPE [21]. C-CPE binds to claudin-4 and decreases TJ barrier function as indicated by a decrease in TER [12]. The C-terminal 30 amino acids of CPE and C-CPE mediate interaction with the CPE receptor and claudin, respectively (a) [22,23,32]. Further analysis shows that the C-terminal 16 amino acids are responsible for the interaction of C-CPE with claudin and for its ability to decrease the TJ barrier function (b) [22,23].

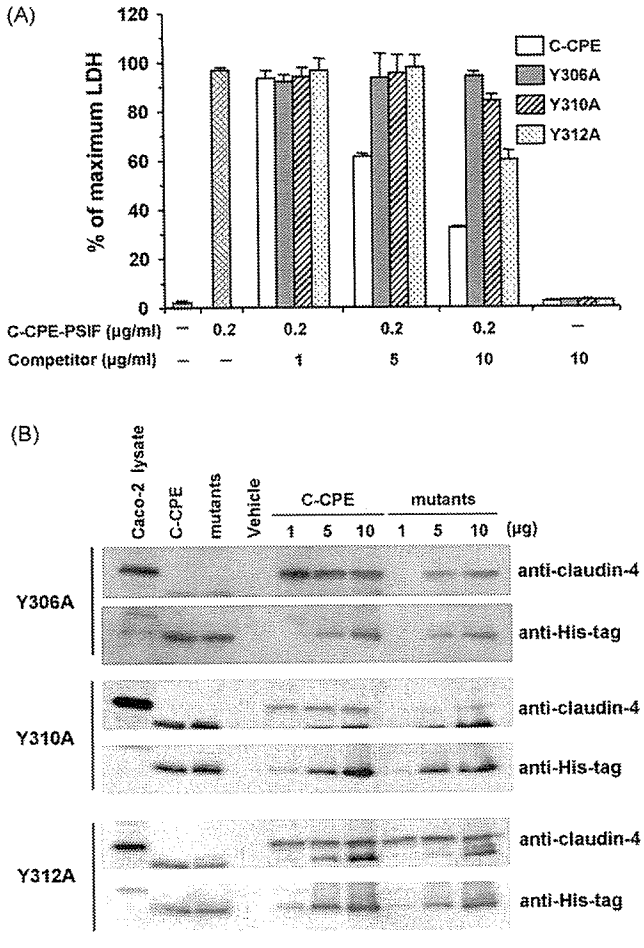

Fig. 2 – Effects of Tyr306, Tyr310 and Tyr312 on the interaction of C-CPE with claudin-4. (A) Competitive inhibition of C-CPE-PSIF-induced cytotoxicity by mutants of C-CPE. Claudin-4-expressing L (CL4/L) cells were pretreated with the indicated concentrations of C-CPE or C-CPE mutants for 1 h. The cells were then incubated with 0.2 μg/ml of C-CPE-PSIF. After 36 h, LDH release was assessed using a commercially available kit. The results are representative of three independent experiments, and the values are means ± S.D. ($n = 3$). (B) Pull-down assay. Confluent Caco-2 cells were harvested and lysed in lysis buffer. The lysate (10 μg) was incubated with vehicle, C-CPE, or mutants of C-CPE for 30 min at 37 °C. After addition of Ni-resin, the lysate was incubated for 3 h at 4 °C. The resin was then precipitated, and the bound proteins were analyzed by SDS-PAGE followed by Western blotting using anti-claudin-4 or anti-His-tag antibodies. The lanes containing Caco-2 lysates (10 μg), C-CPE (1 μg), and mutants of C-CPE (1 μg) were positive controls for claudin-4, C-CPE, and mutants of C-CPE, respectively. The results are representative of three independent experiments.

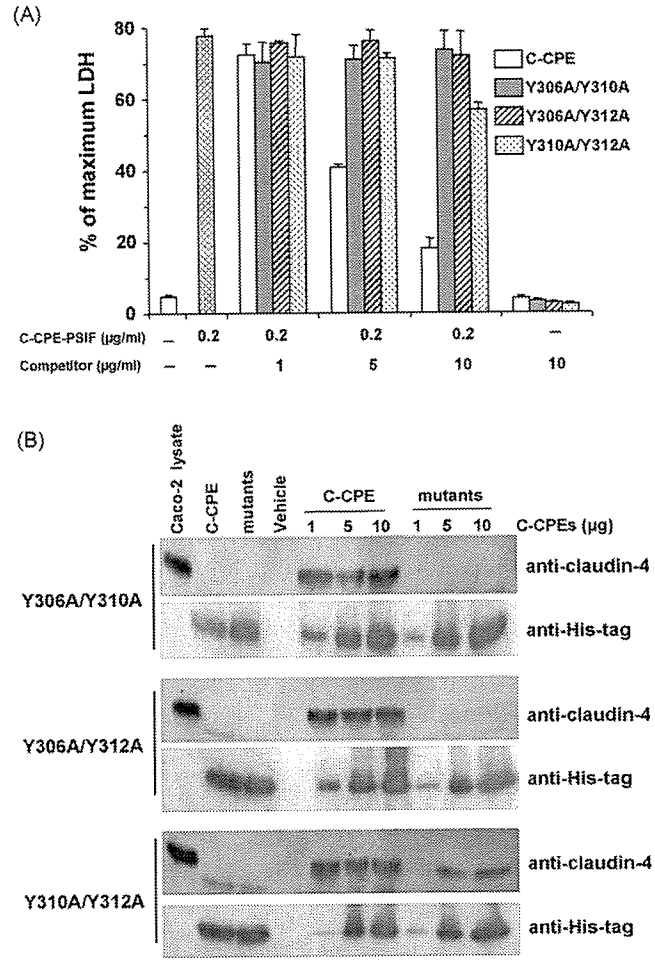

Fig. 3 – Interaction of double mutants at residues 306, 310, and 312 of C-CPE with claudin-4. (A) Competition assay. CL4/L cells were incubated with double mutants at the indicated concentration for 1 h and then mixed with C-CPE-PSIF (0.2 μg/ml). After 36 h, LDH release was assessed using a commercially available kit. The results are representative of three independent experiments. Values are means ± S.D. ($n = 3$). (B) Pull-down assay. Caco-2 lysates (10 μg) were incubated with double mutants (Y306A/Y310A, Y306A/Y312A, or Y310A/Y312A) for 30 min at 37 °C. Ni-resin was then added, and the lysate was incubated for 3 h at 4 °C. Next, the resin was precipitated, and the bound proteins were analyzed by SDS-PAGE followed by Western blotting.

whereas the Y312A and Y306A mutants had weaker abilities to enhance absorption ($AUC_{0-4\,h} = 2.0$ and 6.4, respectively). Double mutant Y306A/Y310A had a moderate absorption-enhancing activity, similar to the Y306A mutant. In contrast, the Y306A/Y312A and Y310A/Y312A double mutants lost the ability to enhance absorption. Thus, there are some differences between the effects of some of the mutants on the TER values and jejunal absorption.

3.4. *Effects of triple mutant Y306A/Y310A/Y312A on the ability to bind to claudin-4 and to modulate the TJ barrier function*

Finally, to clarify role of tyrosine residues 306, 310, and 312 in C-CPE function, we mutated all three to alanine, generating the Y306A/Y310A/Y312A triple mutant. To evaluate the

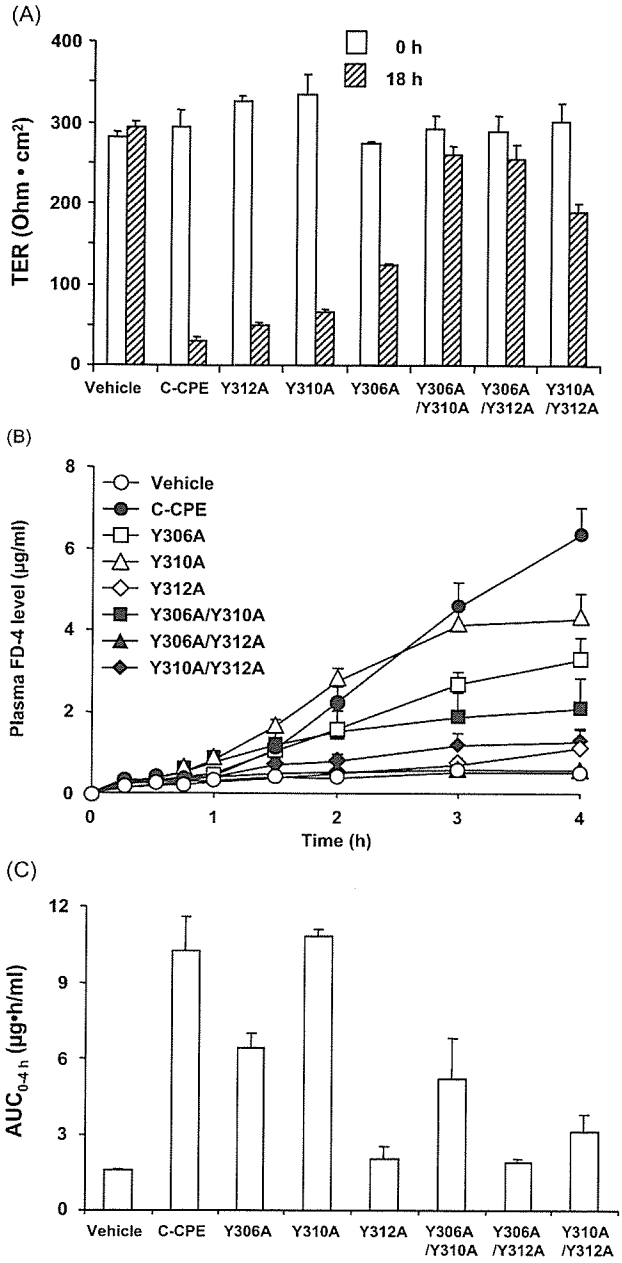

Fig. 4 – Effects of mutants of C-CPE on the TJ barrier function and jejunal absorption. (A) Effects of mutants on the TJ barrier function of Caco-2 monolayer cells. Confluent Caco-2 cells were cultured in Transwell chambers for 10–14 days. When TER values stabilized, C-CPEs were added to the basal side of the Transwell chamber at the indicated concentrations. TER values were measured after 0 and 18 h. Values are means ± S.D. (n = 4). The results are representative of three independent experiments. (B) Effects of mutants on jejunal absorption of FD-4 in rat. Rat jejunum was treated with FD-4 (10 mg/ml) in the presence of vehicle, C-CPE (0.2 mg/ml), or mutant C-CPEs (0.2 mg/ml). The FD-4 levels in plasma collected from the jugular artery were determined at the indicated times. Values are means ± S.E.M. (n = 4). (C) The $AUC_{0-4\ h}$ values were calculated from (B) and are means ± S.E.M. (n = 4). The results shown in (B) and (C) are representative of three independent experiments.

interaction of Y306A/Y310A/Y312A mutant with claudin-4, we performed a competitive assay using C-CPE-PSIF (Fig. 5A) and a pull-down assay using Caco-2 lysates (Fig. 5B). As indicated in Fig. 5A and B, the triple mutant lost the ability to interact with claudin-4. Furthermore, Fig. 5C–E shows that the triple mutant could not modulate TJ barrier function or enhance jejunal absorption.

4. Discussion

Claudins are critical for the barrier function of TJs in epithelia and endothelia [5]. This suggests that modulating the barrier function of claudins can be employed to deliver drugs, and we and Tsukita and colleagues have indeed shown that claudin is a promising target for the development of a drug delivery system [16,17,23,25]. Methods of modulating the barrier function of claudins are, however, very limited, and to our knowledge C-CPE is the only known modulator of a claudin (claudin-4) in TJs [12]. Therefore, we have sought to develop claudin modulators using C-CPE prototype and identified the part of C-CPE that modulates the TJ barrier function [22]. In the current study, we used site-directed mutagenesis and functional assays to clarify the roles of tyrosines 306, 310, and 312 of C-CPE. We found that these three tyrosine residues are critical for binding of C-CPE to claudin-4 and for modulation of the TJ barrier function.

Interestingly, the Y312A mutant had different effects in the pull-down assay using Caco-2 lysates than in the competitive assay using C-CPE-PSIF. Specifically, the Y312A mutant bound to claudin-4 in the pull down assay but it displayed reduced binding in the C-CPE-PSIF competitive assay. These conflicting results could be due to differences in the assay systems or the species of claudin-4: the former assay investigates the interaction of C-CPE with human claudin-4 in cell lysates, whereas the latter assesses the interaction with mouse claudin-4 on the cell membrane [25]. Claudin is tetra-transmembrane protein with two extracellular loop domains [5], and CPE and C-CPE interact with the second of these extracellular loops [22,26]. Examination of the amino acid sequence in the second extracellular loop domain of claudin-4 (EC2cld4) reveals, in fact, that they are different in mouse (GenBank accession no. AF087822; residues 145–163; RDFYNPMVASGQKREMGAS; underline indicates sequence differences with human claudin-4) and human (GenBank accession no. BC000671; residues 142–160; NIIQDFYNPI-VASGQKREM; underline indicates sequence differences with mouse claudin-4) [15].

The Y312A mutant also had different effects between TER assay and *in situ* loop assay. Although the Y312A mutant reduced TER values in Caco-2 monolayer cells, the Y312A mutant did not enhance rat jejunal absorption of FD-4. TER is a marker of tightness in the TJs, but we did not evaluate the permeability of FD-4 in Caco-2 monolayer cells. Several reports indicate that TER values did not reflect permeability of non-electrolytes [27,28]. Therefore, decrease in TER values may be inconsistent with influx of paracellular markers in Caco-2 cells. The amino acids sequences in EC2cld4 are also different in rat (GenBank accession no. NM_001012022; residues 145–163; RDFYNPIVASGQKREMGAS; underline indicates sequence

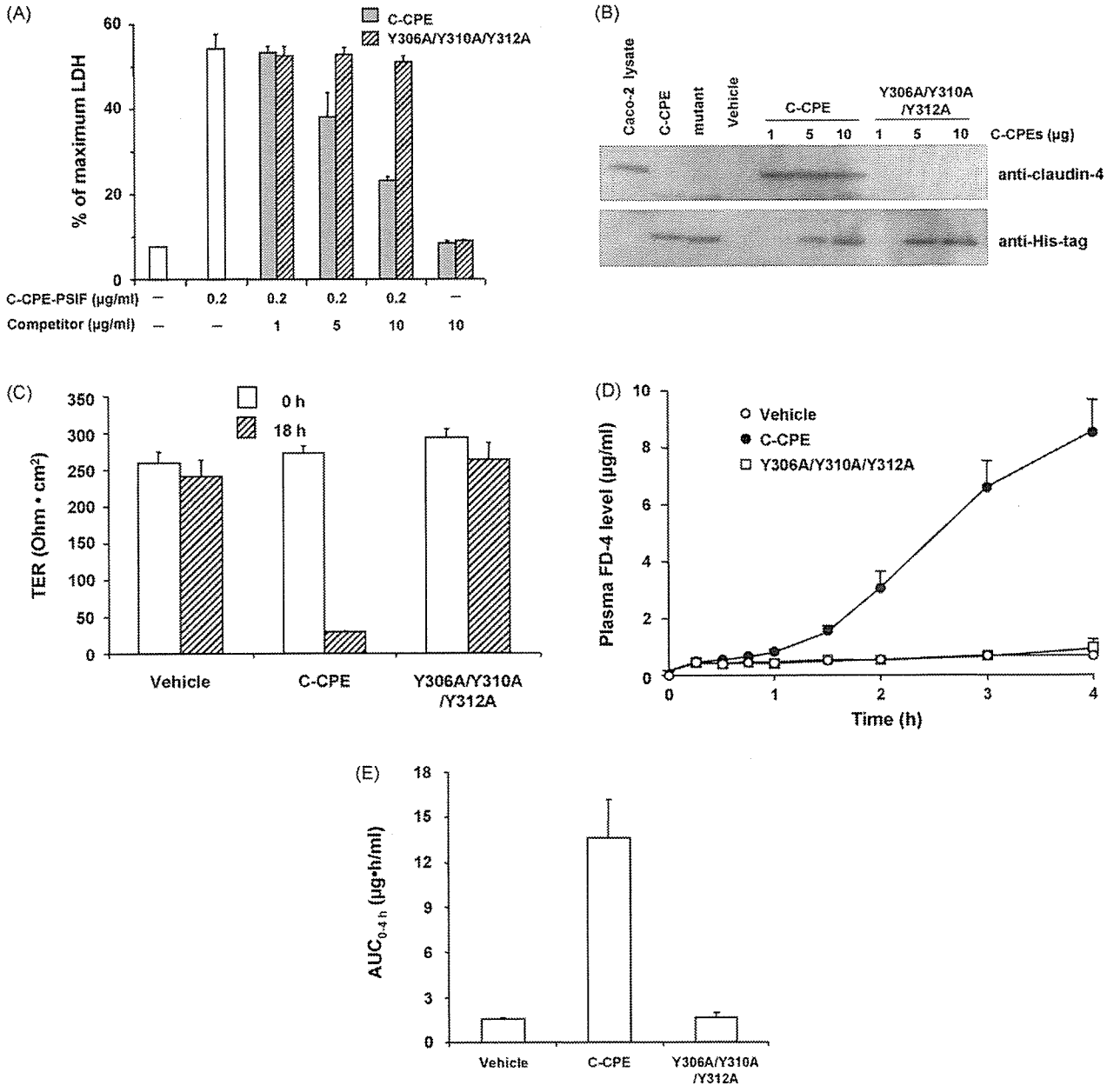

Fig. 5 – Effect of triple mutation on the activities of C-CPE. Interaction of the triple mutant with claudin-4 was examined using competitive assay with C-CPE-PSIF (A) and a pull-down assay (B) as described in Fig. 2A and B, respectively. Effects of the triple mutant on the TJ barrier in Caco-2 monolayer cells (C) and jejunal absorption in rat (D and E) were assayed as described in Fig. 4. The values in (B) and (C) are means ± S.D. ($n = 4$), and those in (D) and (E) are means ± S.E.M. ($n = 4$). The results are representative of three independent experiments.

differences with human claudin-4) and human (GenBank accession no. BC000671; residues 142–160; NIIQDFYNPI-VASGQKREM; underline indicates sequence differences with rat claudin-4) [15]. The differences of EC2cld4 between rat and human may contribute the contradiction of data on Y312A in TER assay and *in situ* loop assay.

We also found that mutation of Tyr310 to alanine reduced binding of C-CPE to claudin-4. This finding agrees with a previous report by Kokai-Kun et al. showing that replacement of Tyr310 with cysteine reduced the ability of CPE to bind to its receptor [24]. Tyr310 may participate in interaction of C-CPE with claudin-4 because the Y310A mutant had the ability to modulate the TJ barrier function in Caco-2 monolayers and to enhance rat jejunal absorption. Whether claudin-4 binding and modulation of the TJ barrier function can be separated is a critical question for the design of future claudin modulators. Mutation of Tyr306 to alanine reduced both activities suggested that they are mediated by the same functional domain on C-CPE. Results with the Y310A mutant, however, indicate that different residues mediate these two functions. In fact, the Y312A mutant did not enhance jejunal absorption in rats (Fig. 4B and C), although it bound claudin-4

in a pull-down assay using rat jejunal lysates (data not shown). Taken together, we cannot exclude the possibility that the two functions of C-CPE are mediated by distinct residues.

The next logical question is whether interaction of C-CPE with claudin-4 is sufficient for disrupting the TJ barrier. Although we did not examine the effect of C-CPE on the level of claudin-4 protein in TJs in the current studies, Sonoda et al. previously reported that treatment of cells with C-CPE causes a decrease in the level of claudin-4 protein in TJs, suggesting that its loss is essential for disruption of the TJ barrier by C-CPE [12]. This loss of claudin-4 could occur through clathrin-mediated endocytosis. Proteins are generally targeted to clathrin-coated vesicles by sorting signal sequences, including YXXØ or EXXXLL (where X is any amino acid and Ø is a bulky hydrophobic residue) [29], and claudin-4 contains an ALGVLL motif at amino acids 92–97 and a YVGW motif at amino acids 165–168 [30]. Indeed, Matsuda et al. showed that the endocytosis of claudins occurs during the remodeling of TJs [31]. The fact that the cytotoxicity of C-CPE-PSIF depends on its uptake into cytosol suggests that, in the CL4/L cells, a molecule bound to claudin-4 can be taken up into the cytosol [25]. In the current study, we found that the Y312A mutant interacts with claudin-4 in a pull-down assay using lysates of rat jejunum but that it does not enhance jejunal absorption. Therefore, Y312A may interact with claudin-4 on rat jejunal epithelial cells but not be taken up into the cytosol.

Single mutation of tyrosines 306, 310, or 312 to alanine resulted in partial reductions of C-CPE activities, whereas double or triple mutations of these tyrosines nearly eliminated the activities. For example, the Y306A/Y310A, Y306A/Y312A, and Y306A/Y310A/Y312A mutants did not bind to claudin-4 or modulate the TJ barrier, whereas the Y310A/Y312A mutant had a partial reduction in binding to claudin-4 and modulation of the TJ barrier function. Taken together, these results show that Tyr306 is a pivotal residue but that it is not the only residue important for the abilities of C-CPE to bind claudin-4 and modulate the TJ barrier. How these three tyrosine residues contribute to the interaction of C-CPE with claudin-4 and to modulation of the TJ barrier is not clear because the three-dimensional structures of CPE and claudin have not been determined. The different effects of C-CPE mutants on C-CPE activities may be due to differences in potency of the mutants or qualitative differences in the mechanism. Regardless, our results suggest that it may be useful to mutate tyrosines 306, 310, and 312 to develop claudin modulators. This information should also be useful for future structural studies on the interaction of CPE with claudin.

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REFERENCES

[1] Schneeberger EE, Lynch RD. Structure, function, and regulation of cellular tight junctions. Am J Physiol 1992;262:L647–61.
[2] Anderson JM, van Itallie CM. Tight junctions and the molecular basis for regulation of paracellular permeability. Am J Physiol 1995;269:G467–75.
[3] Staehelin LA. Further observations on the fine structure of freeze-cleaved tight junctions. J Cell Sci 1973;13:763–86.
[4] Farquhar MG, Palade GE. Junctional complexes in various epithelia. J Cell Biol 1963;17:375–412.
[5] Tsukita S, Furuse M. Pores in the wall: claudins constitute tight junction strands containing aqueous pores. J Cell Biol 2000;149:13–6.
[6] Tsukita S, Furuse M, Itoh M. Multi-functional strands in tight junctions. Nat Rev Mol Cell Biol 2001;2:285–93.
[7] Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita Sa, et al. Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol 1993;123:1777–88.
[8] Saitou M, Fujimoto K, Doi Y, Itoh M, Fujimoto T, Furuse M, et al. Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. J Cell Biol 1998;141:397–408.
[9] Itoh M, Sasaki H, Furuse M, Ozaki H, Kita T, Tsukita S. Junctional adhesion molecule (JAM) binds to PAR-3: a possible mechanism for the recruitment of PAR-3 to tight junctions. J Cell Biol 2001;154:491–7.
[10] Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol 1998;141:1539–50.
[11] Furuse M, Sasaki H, Fujimoto K, Tsukita S. A single gene product, Claudin-1 or -2, reconstitutes tight junction strands and recruits occluding in fibroblasts. J Cell Biol 1998;143:391–401.
[12] Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, et al. *Clostridium perfringens* enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier. J Cell Biol 1999;147:195–204.
[13] Simon DB, Lu Y, Choate KA, Velazquez H, Al-Sabban E, Praga M, et al. Paracellin-1, a renal tight junction protein required for paracellular Mg^{2+} resorption. Science 1999;285:103–6.
[14] Wilcox ER, Burton QL, Naz S, Riazuddin S, Smith TN, Ploplis B, et al. Mutations in the gene encoding tight junction claudin-14 cause autosomal recessive deafness DFNB29. Cell 2001;104:165–72.
[15] Morita K, Furuse M, Fujimoto K, Tsukita S. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci USA 1999;96:511–6.
[16] Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, et al. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol 2002;156:1099–111.
[17] Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 2003;161:653–60.
[18] Furuse M, Sasaki H, Tsukita S. Manner of interaction of heterogeneous claudin species within and between tight junction strands. J Cell Biol 1999;147:891–903.
[19] Anderson JM. Molecular structure of tight junctions and their role in epithelial transport. News Physiol Sci 2001;16:126–30.

[20] McClane BA, Chakrabarti G. New insights into the cytotoxic mechanisms of *Clostridium perfringens* enterotoxin. Anaerobe 2004;10:107–14.
[21] Katahira J, Inoue N, Horiguchi Y, Matsuda M, Sugimoto N. Molecular cloning and functional characterization of the receptor for *Clostridium perfringens* enterotoxin. J Cell Biol 1997;136:1239–47.
[22] Takahashi A, Kondoh M, Masuyama A, Fujii M, Mizuguchi H, Horiguchi Y, et al. Role of C-terminal regions of the C-terminal fragment of *Clostridium perfringens* enterotoxin in its interaction with claudin-4. J Control Rel 2005;108:56–62.
[23] Kondoh M, Masuyama A, Takahashi A, Asano N, Mizuguchi H, Koizumi N, et al. A novel strategy for the enhancement of drug absorption using a claudin modulator. Mol Pharmacol 2005;67:749–56.
[24] Kokai-Kun JF, Benton K, Wieckowski EU, McClane BA. Identification of a *Clostridium perfringens* enterotoxin region required for large complex formation and cytotoxicity by random mutagenesis. Infect Immun 1999;67:5634–41.
[25] Ebihara C, Kondoh M, Hasuike N, Harada M, Mizuguchi H, Horiguchi Y, et al. Preparation of a claudine-targeting molecule using a C-terminal fragment of *Clostridium perfringens* enterotoxin. J Pharmacol Exp Ther 2006;316:255–60.
[26] Fujita K, Katahira J, Horiguchi Y, Sonoda N, Furuse M, Tsukita S. *Clostridium perfringens* enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein. FEBS Lett 2000;476:258–61.
[27] Balda M, Whitney J, Flores C, Gonzalez S, Cereijido M, Matter K. Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. J Cell Biol 1996;134:1031–49.
[28] McCarthy KM, Skare IB, Stankewich MC, Furuse M, Tsukita S, Rogers RA, et al. Occludin is a functional component of tight junction. J Cell Sci 1996;109:2287–98.
[29] Bonifacino JS, Traub LM. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 2003;72:395–447.
[30] Ivanov AI, Nusrat A, Parkos CA. Endocytosis of epithelial apical junctional proteins by a clathrin-mediated pathway into a unique storage compartment. Mol Biol Cell 2004;15:176–88.
[31] Matsuda M, Kubo A, Furuse M, Tsukita S. A peculiar internalization of claudins, tight junction-specific adhesion molecules, during the intercellular movement of epithelial cells. J Cell Sci 2004;117:1247–57.
[32] Hanna PC, Mietzner TA, Schoolnik GK, McClane BA. Localization of the receptor-binding region of *Clostridium perfringens* enterotoxin utilizing cloned toxin fragments and synthetic peptides. J Biol Chem 1991;266:11037–43.

·研究简报·

整合素靶向性病毒载体对肿瘤细胞基因转导的促进作用

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关键词：重组腺病毒；肿瘤细胞；基因表达；整合素

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Gene expression of tumor cells both in vitro and in vivo enhanced by integrin-targeting adenovirus vector

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Abstract: Aim To construct an efficient recombinant viral vector for gene therapy. **Methods** First-generation adenovirus (Ad) vector was modified with the RGD peptide inserted into the fiber. Both *in vitro* and *in vivo* experiments of gene expression in different tumor cells with conventional and recombinant vectors were conducted. RT-PCR was used for detecting the expression of coxackievirus and adenovirus receptor and integrin at the surface of Meth-A cells. **Results** Fiber-mutant adenovirus vector showed a notably enhanced gene expression in A2058, B16BL6, OV-HM, and Meth-A tumor cells compared with that of conventional ones. *In vivo* study carried out using Meth-A tumor-bearing mice also demonstrated that the intra-tumoral injection of recombinant adenovirus induced strong gene expression in these CAR-deficient tumor cells. **Conclusion** The recombinant vector can be a promising one for effective cancer gene therapy.

Key words: recombinant adenovirus vector; tumor cell; gene expression; integrin

在进行基因导入研究及临床试验中，目前采用的载体通常包括了病毒及非病毒两大类[1~3]。对于非病毒载体用于基因转染有许多报道[4,5]。而腺病毒作为基因载体，因其具有的高效转导能力及对分裂或非分裂细胞均能转导等优点而被广泛应用于针对疾病的基因治疗中[6,7]。对腺病毒进入细胞机制进行的深入研究结果发现，腺病毒与细胞结合的第一步是与细胞表面的柯萨奇腺病毒受体（coxsackie adenovirus receptor, CAR）结合，第二步是利用其在五角体基（penton base）中的 RGD 序列和细胞表面的整合素结合，从而促进病毒内吞并利用病毒对溶酶体的逃逸作用及进入核内而实现所携带基因的高效表达[8~10]。因此，如果宿主细胞表面缺乏 CAR，采用野生型腺病毒很难产生高效基因表达，从而丧失其作为病毒载体的最大优点。Curiel等[11]最先采用基因重组手段改变腺病毒的纤维突起（fiber knob），提高对采用野生型腺病毒难以达到高效转导细胞的转导效率。已有报道证明采用 Ad5/35纤维的复合体可以产生腺病毒的 CAR非依赖性的基因导入，说明新型腺病毒载体可以无需 CAR的参与而

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对细胞进行导入,为设计新的导入途径提供了依据[12]。Yotnda等[13]对病毒纤维部分用聚赖氨酸K21等进行修饰,也提高了对白血病细胞的转导效率。对于改变载体靶向性,采用具有整合素靶向性的RGD序列对基因载体进行修饰引起了广泛重视。熊小兵等[14]采用RGD类似物修饰非病毒载体携载抗肿瘤药物,显著增加了药物向肿瘤细胞内的传递。本文构建了在纤维突起上编码有RGD-4C序列的重组腺病毒(AdRGD),并认为这将会使得病毒在CAR低表达细胞上直接进入第二步骤的结合,即插入HI环中的RGD与整合素直接结合从而产生基因的有效导入。

材料与方法

细胞与动物 人黑素瘤细胞A2058,鼠黑素瘤细胞B16BL6及人胚肾细胞HEK293均购自JCRB细胞库(东京,日本);鼠纤维肉瘤细胞Meth-A和鼠卵巢癌细胞OV-HM由大阪大学医学部藤原大美博士提供。A2058细胞和HEK 293细胞均培养于含10%小牛血清和抗生素的DMEM培养基中,B16BL6细胞培养于含7.5%小牛血清和抗生素的DMEM培养基中,Meth-A和OV-HM细胞则均培养于含10%小牛血清和抗生素的RPMI 1640培养基中。6~8周龄的雌性BALB/c小鼠购自SLC公司(静冈,日本)。

重组腺病毒构建 本实验所用的复制缺陷腺病毒均由去除E1和E3的血清5型腺病毒作为骨架。重组腺病毒系采用体外两步法将整合素靶向的RGD序列插入到纤维突起的HI环中[15,16]。另将报告基因荧光素酶(Luc)和增强绿荧光蛋白(EGFP)的基因编码入E1区域。野生型和重组腺病毒的构造见图1。病毒载体均采用以前报道的方法进行增殖及纯化[17],即在HEK293细胞内使病毒增殖,回收后采用2次氯化铯梯度超离心,经透析后保存于-80 ℃。病毒感染力采用在293细胞的plaque-forming assay来测定,病毒颗粒数则采用吸收度测定[18]。

腺病毒对各种肿瘤细胞的体外基因转导 将A2058, B16BL6, OV-HM和Meth-A细胞2 $\times10^3$个接种于96孔板中,分别加入1 250, 2 500, 5 000和10 000病毒子/细胞的编码荧光素酶的野生型病毒(Ad-Luc)或重组病毒(AdRGD-Luc),培养48 h后收集细胞,清洗,裂解后采用Luciferase assay system(Promega)和Microlumat Plus LB96(Perkin Elmer)测定荧光素酶活性。

腺病毒对纤维肉瘤的在体转导 将Meth-A细胞1 $\times10^6$个接种于6~8周龄的BALB/c小鼠皮下,一周后将2 $\times10^8$ pfu(plaque forming unit)/肿瘤的编码增强绿荧光蛋白的野生型和重组腺病毒分别注入小鼠肿瘤内,7 d后回收肿瘤,经不同浓度的蔗糖溶液梯度浸泡后,液氮速冻,制备得到6 μm的冰冻切片,在荧光显微镜(Olympus)下观察绿荧光蛋白的表达。

纤维肉瘤细胞表面相关受体的确认 逆转录酶联聚合反应(RT-PCR)被用于检测纤维肉瘤细胞Meth-A的表面分子如CAR,α_v,β_3和β_5整合素的表达。各个表面分子的引物序列及反应条件见表1。细胞的总RNA采用TRI试剂(Sigma)抽提,逆转录采用经DNase处理的总RNA 2 μL,加入RT缓冲液4 μL,DTT 1 μL, 2 mmol·L^{-1} dNTP 1 μL, 0.5 μg·

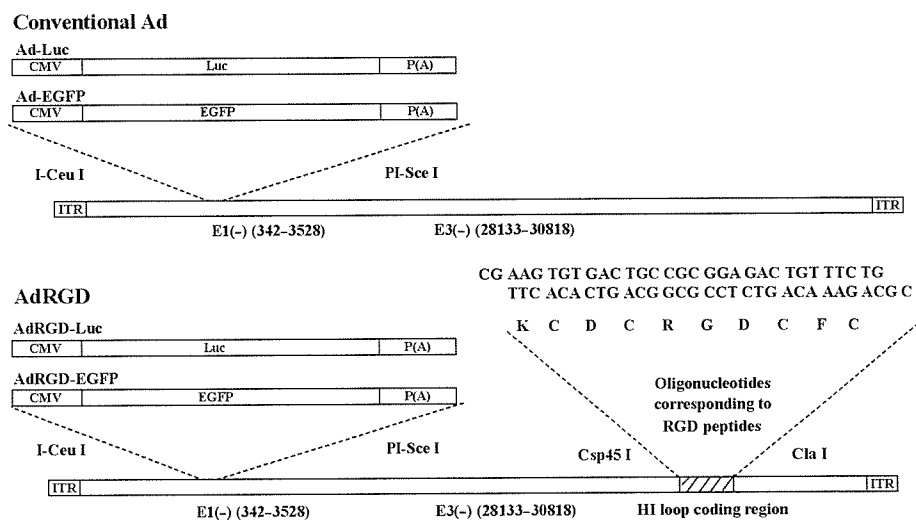

Figure 1 Representative of the construction of recombinant adenovirus vectors

Table 1 Primer sequences used for RT-PCR amplification

Gene	Primer sequence (5′-3′)	Denaturation	Annealing	Extension	Cycle No.	Product size/bp
CAR	(F) TGATCATTTTGTATTCTGGA	for 45 s	for 60 s	for 90 s	30	211
	(R) TTAACAAGAACGGTCAGCAG	at 94 ℃	at 50 ℃	at 72 ℃		
α_v Integrin	(F) CCAGCCTGGGATTGTAGAAG	for 45 s	for 60 s	for 90 s	40	125
	(R) ACTCCAGTGGGTCATCTTTG	at 94 ℃	at 53 ℃	at 72 ℃		
β_3 Integrin	(F) TCTGGCTGTGAGTCCTGTGT	for 45 s	for 60 s	for 90 s	40	135
	(R) GCCTCACTGACTGGGAACTC	at 94 ℃	at 55 ℃	at 72 ℃		
β_5 Integrin	(F) TCGTGTGAAGAATGCCTGTT	for 45 s	for 60 s	for 90 s	30	146
	(R) GCTGGACTCTCAATCTCACC	at 94 ℃	at 53 ℃	at 72 ℃		
β-Actin	(F) TGTGATGGTGGGAATGGGTCAG	for 45 s	for 60 s	for 120 s	20	514
	(R) TTTGATGTCACGCACTATTTCC	at 94 ℃	at 60 ℃	at 72 ℃		

μL^{-1} dT 1 μL, RNase抑制剂 1 μL和 SuperScript III (Invitrogen) 1 μL进行反应。50 ℃反应 1 h。然后取 cDNA 2 μL进行扩增 (条件见表 1)。PCR产物在 1.5%琼脂糖凝胶上进行电泳,溴化乙啶处理后检测荧光。

结果与讨论

作为一种被广泛使用的基因治疗载体,腺病毒亦存在许多缺陷,如免疫原性强、体内滞留时间短等,有人构建了几乎删除全部基因组的所谓无肠腺病毒 (gutless adenovirus)以试图克服其免疫原性[19],以及利用高分子如 PEG修饰以提高病毒在血液循环中的滞留性和避免中和抗体的作用等[20]。一般认为腺病毒的优势在于对细胞有高效转导能力,但通过研究发现腺病毒对某些细胞,如表面 CAR低表达或几乎不表达的细胞并不能产生有效转导。本研究即针对此问题,采用靶向整合素的 RGD插入至 HI环上构建了重组腺病毒,对 4种人和鼠的肿瘤细胞进行的体外转导发现与野生型腺病毒相比,重组腺病毒的转导效率提高了十倍至数十倍不等,而且编码基因荧光素酶的活性呈剂量依赖性 (图 2),从而证明所构建载体在体外的有效性。

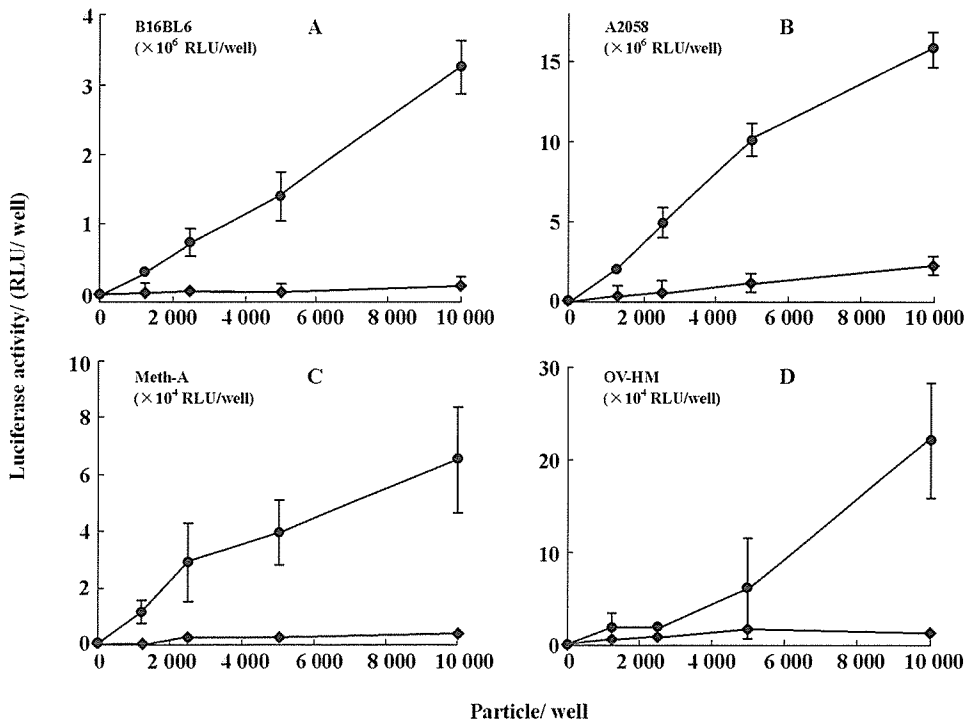

Figure 2 Luciferase expression of Ad-Luc or AdRGD-Luc transfected tumor cells 2×10^3 of B16BL6 melanoma cells, A2058 melanoma cells, OV-HM ovarian carcinoma cells, and Meth-A fibrosarcoma cells per well in 96-well plate were transfected with Ad-Luc (◆) or AdRGD-Luc (●) separately at indicated viral particles/cell for 48 hours, then cells were washed, collected and luciferase activity was measured. Data are presented as mean ± SD of relative light units (RLU) /well determined from three experiments

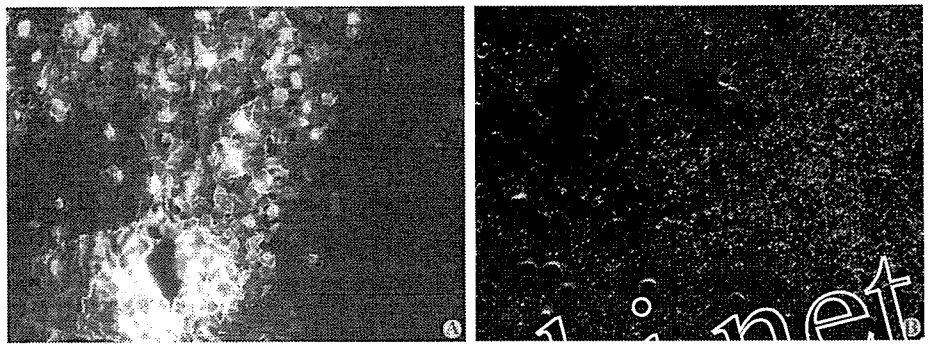

Figure 3 Gene expression in Meth-A tumor via intratumoral injection. 1×10^6 Meth-A cells were inoculated intradermally and after 1 week, (A) 2×10^8 pfu/tumor AdRGD-EGFP or (B) 2×10^8 pfu/tumor Ad-EGFP were intratumoral injected. Tumor nodes were harvested and sectioned using cryostat seven days later and the fluorescence was observed with microscope (×400)

为了评价所构建的载体在荷瘤动物肿瘤内的转导效果,作者将编码增强绿荧光蛋白的野生型及重组腺病毒注入小鼠 Meth-A 纤维肉瘤瘤体内,1周后观察报告基因的表达。结果 (图 3)显示采用野生型腺病毒产生的荧光非常弱,而重组病毒导致了绿荧光蛋白的高效表达,从而证明所构建载体的在体活性及其有效性。

对黑素瘤 A2058和 B16BL6等细胞的表面分子进行测定已证明其表面 CAR的低表达。本研究在体内外都证实重组腺病毒对 Meth-A 纤维肉瘤的高效转导,而 RT-PCR的结果也进一步表明 Meth-A 细胞表面几乎没有或仅有少量 CAR的表达,但存在 $\alpha_v\beta_5$ 整合素的高表达 (图 4),从而提示重组载体可以通过与整合素结合而进入到细胞内,实现基因的高效表达。

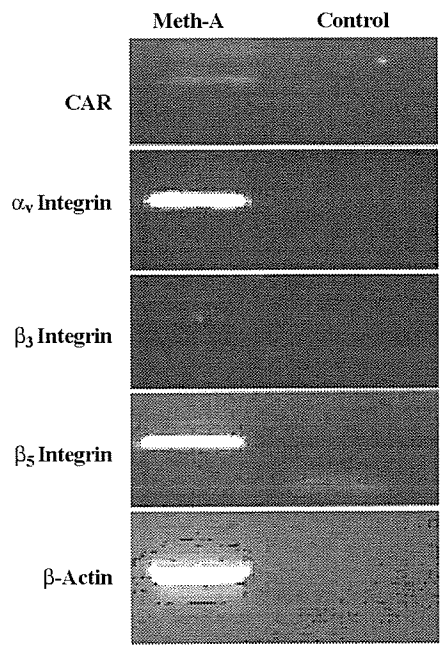

Figure 4 RT-PCR analysis of CAR, α_v-integrin, β_3-integrin, and β_5-integrin in Meth-A fibrosarcoma. Total RNA was prepared from Meth-A fibrosarcoma cells and then RT-PCR was performed as described in Table 1

结论

在 HI环中插入整合素靶向性的 RGD序列,构建获得的重组腺病毒在体外显示了对 4种人和鼠肿瘤细胞的高效转导,在 CAR低表达的 Meth-A 荷瘤小鼠瘤内注射亦证明此载体的有效性。从而为实现肿瘤的高效基因治疗提供了一个具有良好前景的载体。

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References

[1] Lechardeur D, Verkman AS, Lukacs GL. Intracellular routing of plasmid DNA during non-viral gene transfer [J]. Adv Drug Deliv Rev, 2005, 57: 755 - 767.

[2] Louz D, Bergmans HE, Loos BP, et al. Cross-species transfer of viruses: implications for the use of viral vectors in biomedical research, gene therapy and as live-virus vaccines [J]. J Gene Med, 2005, 7: 1263 - 1274.

[3] Chen HL, Liang WQ, Shao JB, et al. Preparation of cationic liposomes and its role in enhancing cellular uptake of antisense oligonucleotides [J]. Acta Pharm Sin (药学学报), 2004, 39: 72 - 76.

[4] Feng M, Li P. PEI-PMMA cationic nanoparticles as carriers for gene transfer [J]. Acta Pharm Sin (药学学报), 2005, 40: 893 - 897.

[5] Wang WX, Liang WQ, Song BW, et al. Cellular uptake behavior of antisense oligodeoxynucleotides polymethacrylate submicroparticles [J]. Acta Pharm Sin (药学学

报), 2005, 40: 950 - 953.

[6] Gao JQ, Tsuda Y, Katayama K, et al. Anti-tumor effect by interleukin-11 receptor alpha-locus chemokine/CCL27, introduced into tumor cells through a recombinant adenovirus vector [J]. Cancer Res, 2003, 63: 4420 - 4425.

[7] Campbell EM, Hope TJ. Gene therapy progress and prospects: viral trafficking during infection [J]. Gene Ther, 2005, 12: 1353 - 1359.

[8] Bergelson JM, Cunningham JA, Droguett G, et al. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5 [J]. Science, 1997, 275: 1320 - 1323.

[9] Wickham TJ, Mathias P, Cheresh DA, et al. Integrins $\alpha_v\beta_3$ and promote adenovirus internalization but not virus attachment [J]. Cell, 1993, 73: 309 - 319.

[10] Wickham TJ, Filardo EJ, Cheresh DA, et al. Integrin $\alpha_v\beta_5$ selectively promotes adenovirus mediated cell membrane permeabilization [J]. J Cell Biol, 1994, 127: 257 - 264.

[11] Dmitriev I, Krasnykh V, Miller C, et al. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxackievirus and adenovirus receptor-independent cell entry mechanism [J]. J Virol, 1998, 72: 9706 - 9713.

[12] Yotnda P, Onishi H, Heslop HE, et al. Efficient infection of primitive hematopoietic stem cells by modified adenovirus [J]. Gene Ther, 2001, 8: 930 - 937.

[13] Yotnda P, Zompeta C, Heslop HE, et al. Comparison of the efficiency of transduction of leukemic cells by fiber-modified adenovirus [J]. Hum Gene Ther, 2004, 15: 1229 - 1242.

[14] Xiong XB, Huang Y, Lu WL, et al. Preparation of doxorubicin-loaded stealth liposomes modified with RGD mimetic and cellular association *in vitro* [J]. Acta Pharm Sin (药学学报), 2005, 40: 1085 - 1090.

[15] Mizuguchi H, Koizumi N, Hosono T, et al. A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob [J]. Gene Ther, 2001, 8: 730 - 735.

[16] Mizuguchi H, Kay MA, Hayakawa T. *In vitro* ligation-based cloning of foreign DNAs into the E3 and E1 deletion regions for generation of recombinant adenovirus vectors [J]. Biotechniques, 2001, 30: 1112 - 1114.

[17] Okada N, Gao JQ, Sasaki A, et al. Anti-tumor activity of chemokine is affected by both the kinds of tumors and the activation state of host's immune system: implications for chemokine-based cancer immunotherapy [J]. Biochem Biophys Res Commun, 2004, 317: 68 - 76.

[18] Maizel JV Jr, White DO, Scharff MD. The polypeptides of adenovirus I Evidence for multiple protein components in the virion and a comparison of type 2, 7A, and 12 [J]. Virology, 1986, 36: 115 - 125.

[19] Burroughs KD, Kayda DB, Sakhuja K, et al. Potentiation of oncolytic adenoviral vector efficacy with gutless vectors encoding GMCSF or TRAIL [J]. Cancer Gene Ther, 2004, 11: 92 - 102.

[20] Eto Y, Gao JQ, Sekiguchi F, et al. PEGylated adenovirus vectors containing RGD peptides on the tip of PEG show high transduction efficiency and protection ability from neutralizing antibodies [J]. J Gene Med, 2005, 7: 604 - 612.

REVIEW

Fusogenic liposomes and their suitability for gene delivery

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A novel carrier system to deliver large exogenous molecules into the cytosol has advantages for efficient delivery compared with traditional transfection techniques. We previously prepared a fusogenic liposome (FL) system by fusing phospholipid-based conventional liposomes with ultraviolet inactivated Sendai virus, which possess membrane fusion activity derived from the Sendai virus accessory protein. The FLs deliver their contents rapidly and directly into the cytoplasm rather than by endocytosis via membrane fusion without cytotoxicity. This FL-mediated cytoplasmic delivery technique is a powerful tool with the potential for wide use in the field of gene regulation and for the development of gene therapy. This review focuses on the suitability of FLs as a versatile and effective gene delivery system.

Keywords: antisense oligonucleotide, DNA vaccine, fusogenic liposome, gene therapy, nanoparticle, Sendai virus

future medicine

The potential influence of gene regulation in life science and pharmaceutical research is tremendous and the information acquired from this research is useful and valuable for the development of gene-based drugs. The alteration of gene expression levels by introducing or silencing genes is a useful method for obtaining a better understanding of cellular functions and will facilitate the development of gene-based drugs. Furthermore, improving the tools to influence cells will enable us to obtain more precise information regarding cells and the optimization of gene therapy, thereby improving our knowledge and treatment of human diseases.

Gene regulation (expression and knockdown and/or knockout) are key biological processes. The control of these cell functions by delivering nucleic acids such as plasmids, antisense oligonucleic acids (AODNs) and short interfering (si)RNAs is important for understanding cellular functions and for the potential treatment of many human diseases [1–3]. Cells and cellular compartments are separated by biological membranes that prevent the influx and efflux of solutes from cells and organelles. Cell membranes are one of the major obstacles encountered when altering gene expression and various techniques have been developed to translocate biologically active molecules across these barriers *in vitro* and *in vivo*.

There are several disadvantages associated with many of the techniques currently available. For example, microinjections are impractical for *in vivo* use since these methods require disruption of the cell membrane before substances can be introduced into the cell. Other methods, such as liposome encapsulation and receptor-mediated endocytosis, are limited by their low delivery yield [4]. As a negative surface charge is a common feature of liposomes, these liposomal carriers are generally taken up by target cells via endocytosis and almost all of the encapsulated nucleic acids are degraded in the lysosomes [5,6]. Thus, a novel strategy to deliver nucleic acids to the cytosol is desirable, and several efficient techniques, such as cationic liposomes, are being developed by various groups.

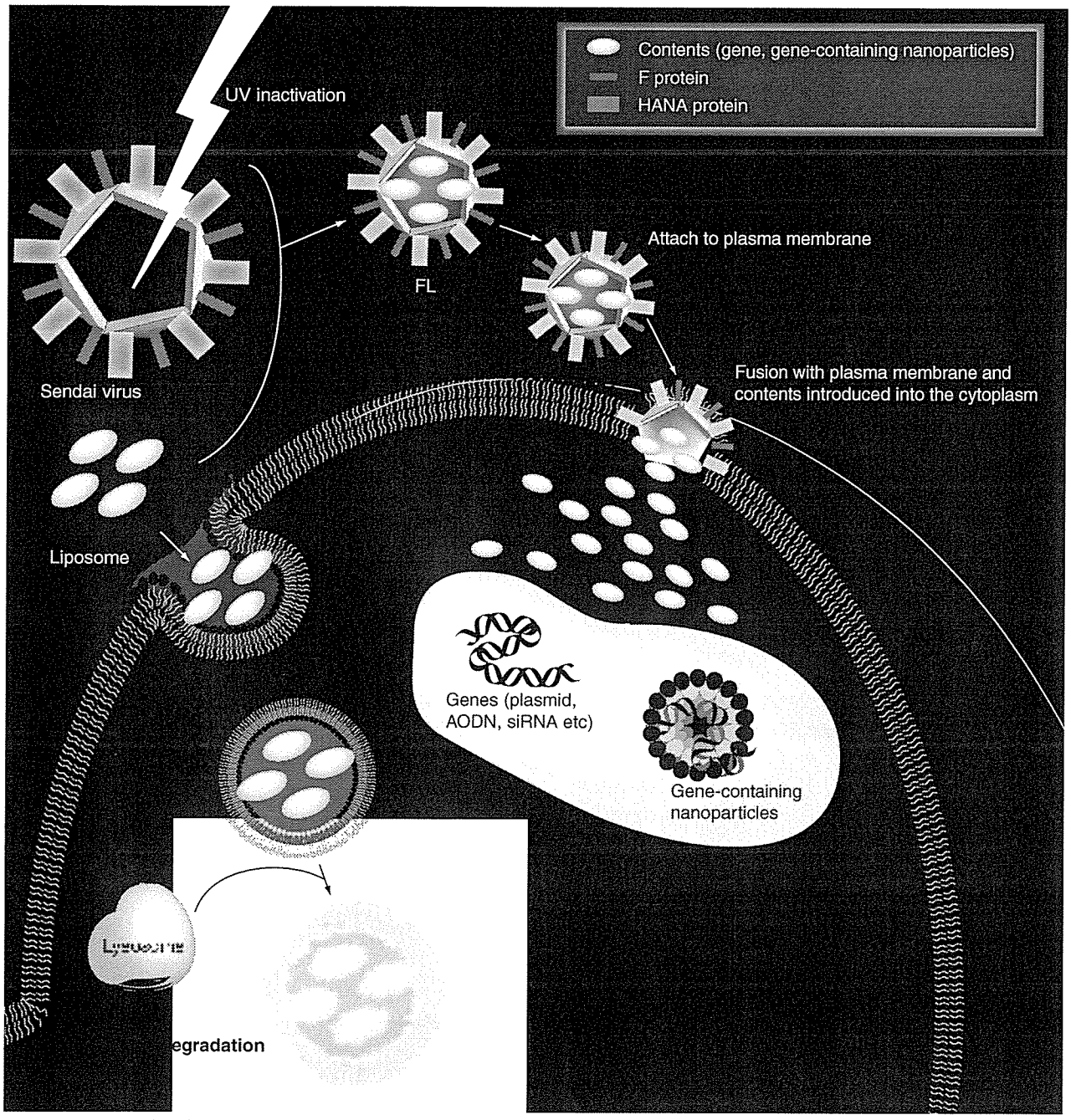
We developed a hybrid delivery system utilizing fusogenic liposomes (FLs), which are composed of conventional liposomes and Sendai virus. Previous studies indicated that FLs deliver encapsulated contents into the cytosol efficiently, rapidly and directly [7–15]. Early reports demonstrate that membrane fusion occurs via Sendai virus accessory proteins (fusion [F] and hemagglutinin–neuraminidase [HANA] proteins) and these activities are energy-independent and unaffected by various endocytosis inhibitors [16]. These features make FLs excellent and powerful tools as vectors for biologically active molecules in gene regulation (**Figure 1**). This review describes the historical and biological features of FLs, and their application to the field of gene therapy, such as cytokine gene therapy and DNA vaccine development. Additionally, to regulate the intracellular gene-release kinetics, we recently developed cytosolic nanoparticle (NP) delivery methods combined with FLs. The recent progress of these investigations is described in this review.

Characterization of fusogenic liposomes

Historical overview

The Sendai virus belongs to *Paramyxoviridae* and has a negative strand genomic RNA. Sendai virus possesses two major proteins; HANA and

Figure 1. Schematic representation of the application of the fusogenic liposome system as a vehicle for gene delivery.



AODN: Antisense oligodeoxynucleotides; F: Fusion; FL: Fusogenic liposome; HANA: Hemagglutinin–neuraminidase; siRNA: Short interfering RNA; UV: Ultraviolet.

F proteins, on its surface membrane. HANA proteins are important for binding to the receptor (sialic acid) on the cell surface [17]. After binding of HANA and its receptor, F proteins interact with the lipid bilayer of the cell membrane to induce cell fusion [18,19]. Although another HANA-dependent intermediate stage of Sendai virus-mediated membrane fusion has been reported [20], the mechanisms by which Sendai virus infects cells are not clearly understood.

Previously, Okada and colleagues reported that the Sendai virus fuses with not only the cell membrane but also with liposomes at 37°C, and this process involves interactions between the Sendai virus envelope protein and

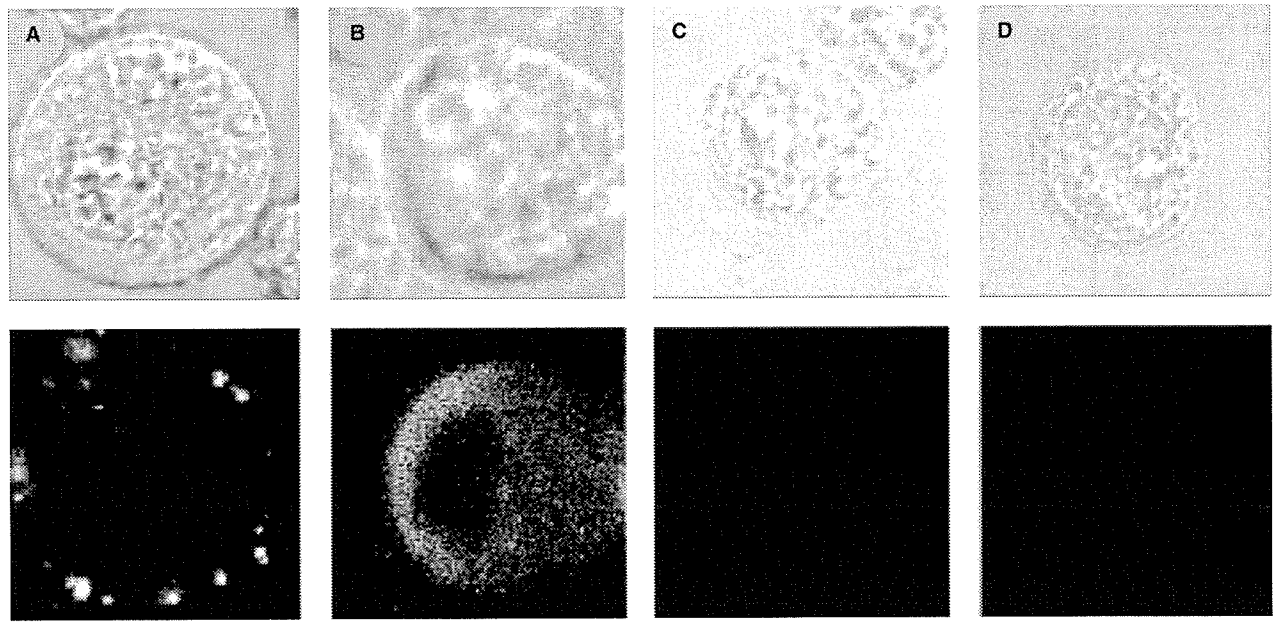
liposomes [20,21]. In addition, they reported that F proteins, but not HANA proteins, are essential for the fusion step between liposomes and Sendai virus [22]. From these reports, the Sendai virus receptor (sialic acid) is not required for the fusion step because sialic acid does not exist on liposomes. This unique fusion mechanism between Sendai virus and the liposomes enables the hybrid Sendai virus–liposome vehicle to fuse with mammalian cell membranes [20].

Previously, we developed a method for FL preparation [7–9]. After a fusion reaction of ultraviolet inactivated Sendai virus and conventional liposomes under optimal conditions (neutral pH and 37°C), FL can be purified from the unreacted Sendai virus and liposomes by discontinuous (12, 30 and 50%) sucrose gradient centrifugation (77000 × g, 2 h and 4°C). As a result, small FL can be purified when unilamellar liposomes (diameter: 300 nm) are fused with the Sendai virus (diameter: 300 nm). From dynamic light scattering analysis, the estimated diameter of each FL is approximately 380 nm, suggesting that a single Sendai virus fuses with a single liposome. In addition, electron microscopic observation revealed that FLs possess a spike structure on the surface, similar to that of the Sendai virus [8,9,16].

Cytoplasmic delivery of macromolecules using FLs

The unique characteristic of FLs is that they fuse with a wide variety of mammalian cell membranes and deliver their contents, which are encapsulated in liposomes, into the cytoplasm. To visualize the cytoplasmic delivery of macromolecules by FLs, we performed confocal microscopic analyses [16]. EL4 lymphoma cells were incubated at 37°C for various times with Alexa Fluor 488-conjugated bovine serum albumin (Alexa-BSA) that was either free or entrapped in either FLs or conventional liposomes. After 10 min of incubation, Alexa-BSA from FL was observed around the cells (Figure 2A). After 3 h of further incubation, these cells demonstrated diffuse cytosolic staining (Figure 2B). In contrast, neither surface staining after 10-min incubation nor diffuse staining after 3-h incubation was observed in the EL4 cells incubated with either free Alexa-BSA or Alexa-BSA entrapped in simple liposomes (Figure 2C & D). Furthermore, an endocytosis inhibitor did not affect the delivery capacity of the FLs [14,16,23]. These findings indicate that FL delivered their contents into the cytoplasm via an endocytosis-independent pathway, whereas conventional liposomes were taken up by endocytosis.

Figure 2. Fusogenic liposome-mediated cytosolic delivery of encapsulated macromolecules.



EL4 cells were incubated with fusogenic liposomes containing Alexa Fluor 488-conjugated bovine serum albumin (Alexa-BSA) for 10 min **(A)** or 3 h **(B)**, conventional liposomes containing Alexa-BSA **(C)** or Alexa-BSA solution **(D)** for 3 h. After incubation, the cells were washed and analyzed by confocal microscopy. Diffuse staining, representing cytosolic macromolecules, was observed only in EL4 cells incubated with FL containing Alexa-BSA for 3 h **(B)**.

Feasibility of FLs as gene delivery vehicles

Characterization of FLs as gene delivery vehicles

Gene therapy was recently recognized as an effective approach for obtaining clinical benefits from foreign therapeutic genes expressed in affected tissues. Although these approaches are promising, therapeutic genes (e.g., plasmids, AODNs and siRNAs) are rapidly and easily degraded by extracellular or lysosomal enzymes [5,6]. A promising approach to overcome these obstacles is the direct delivery of genes into the cytoplasm. In this context, FLs might also be effective for gene therapy. To clarify the potential of FLs as gene delivery vehicles, we first analyzed the gene expression efficiency of FLs containing plasmids [8,9,24]. *In vitro* gene expression analysis clearly demonstrated that FLs transferred encapsulated plasmids into target cells more rapidly compared with cationic liposomes [24]. In addition, the gene expression level was higher than that resulting from the use of cationic liposomes, even under high serum concentrations. Consistent with the *in vitro* results, FLs introduced encapsulated luciferase-expression vectors *in vivo* (Table 1). Further analysis indicated that the FL-mediated effective gene transfer system possesses high potential as a tool for *in vivo* cancer gene therapy [25]. These findings clearly indicated that FLs can function as novel and efficient gene delivery vehicles.

Application of FL systems as DNA vaccine carriers

Genetic immunization using plasmid (p)DNA-encoding antigens from bacteria, viruses and cancers leads to protective cell-mediated (T-helper [Th]1) and humoral (Th2) immunity [26]. This system has some practical advantages over conventional vaccines, such as safety, stability, cost–effectiveness for manufacturing and ease of modifying and customizing the gene sequence to produce the desired type of recombinant protein for expression *in vivo*. Although naked DNA vaccines (i.e., pDNA in saline) are effective in small animal models [27–29], general results from large animal and nonhuman primate studies have been disappointing due to suboptimal immune responses, despite the use of multi-milligram doses of naked pDNA [30]. Therefore, the development of adjuvants and excipients to increase immune responses to DNA vaccines has become an active area of research. The potential for genetic immunization to exert an effective antigen-specific immune response is directly related to both the level of expression of the encoded protein and the immunomodulatory activity generated by DNA vaccine formulations [31–33].

Two main reasons for the ineffectiveness of genetic immunization have been proposed. First, pDNAs are generally unstable *in vivo*, as mentioned previously [34–36]. In addition, a small amount of undegraded pDNA is taken up via endocytosis by neighboring cells around the injection site, including antigen-presenting cells. Endocytosed pDNAs are, thus, generally degraded in intracellular compartments such as endosomes or lysosomes. Consequently, extremely low levels of pDNAs that escape from these host factors express their encoded antigens, resulting in inefficient induction of antigen-specific immunity. Owing to the extracellular and intracellular degradation and low immunogenicity of naked pDNAs, genetic immunization exhibits poor performance when administered by routes other than intramuscular ones [30].

To address these issues, several DNA-particulate approaches have been evaluated [37]. Recently, liposomes and microscale or nanoscale particles were tested for genetic vaccination [37–43]. However, due to the degradation of pDNA by antigen-presenting cells a novel carrier-mediated approach to improve the potency of DNA vaccines is required. Since FLs efficiently deliver encapsulated genes into the cytoplasm and the introduced genes are expressed efficiently, we hypothesized that FLs would be as effective as DNA vaccine vehicles to deliver antigen-encoding plasmids. In this context, we examined the vaccine efficiency of FLs containing ovalbumin (OVA)-encoding plasmids (pOVAs) [10,12]. Initially, using reverse transcription-PCR analysis, we verified the

Table 1. Comparison of *in vivo* gene transfer activity of fusogenic liposomes and cationic liposomes.

Carrier	DNA dose (μg/mouse)	Luciferase activity (RLU/10^7 cells)
Fusogenic liposome	1.5	8013 ± 809
Cationic liposome	1.5	4.9 ± 0.7
	40	92.2 ± 18.5

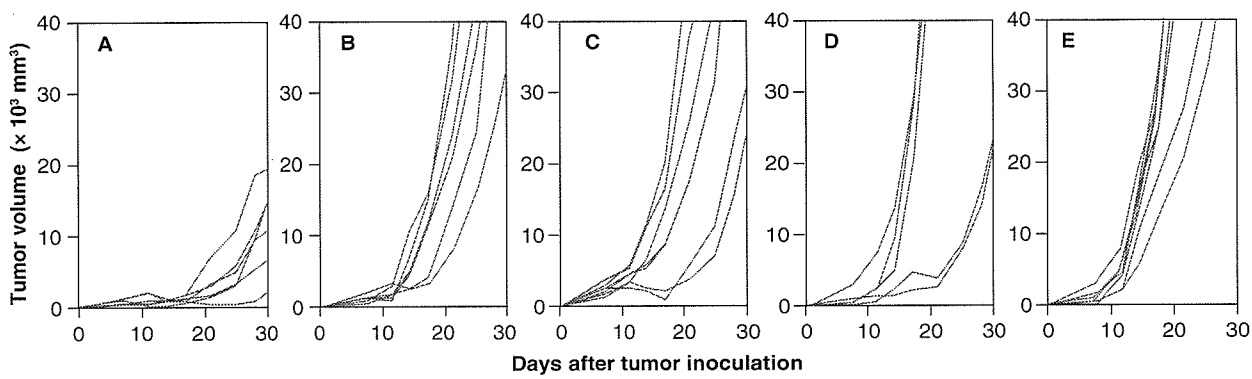
S-180 tumor cells were intraperitoneally injected into male ddY mice at day 0. At 5 days, luciferase expression plasmids encapsulated in fusogenic liposomes or complexed with cationic liposomes were given intraperitoneally. At 7 days after injection, S-180 cells were recovered and luciferase activity was measured. Data represent the mean ± standard deviation of three mice.
RLU: Relative light units.

expression of OVA mRNA transcribed from the pOVAs, used for DNA vaccination. We performed *in vitro* transfection studies to assess the OVA proteins expressed in a transfected mouse macrophage cell line (IC-21) and fibroblasts (L cells). Surprisingly, only 10 min after transfection, remarkably higher OVA mRNA expression was detected in IC-21 and L cells treated with FL than in those treated with other transfection techniques. For example, OVA expression was lower for a commercially available cationic lipid-based gene delivery method (Lipofectin™) than for FL-mediated transfection. Next, we investigated whether FL-mediated DNA vaccination induced antigen-specific antitumor immunity. To determine the effects of FL-mediated vaccination, we analyzed tumor growth and survival rate after vaccination in the E.G7-OVA model. A total of 4 weeks after the last immunization, mice were intradermally challenged with 10^6 live E.G7-OVA cells in the abdomen. High concentrations of naked pOVA, 5 µg of pOVA/Lipofectin complexes or pOVAs encapsulated in conventional liposomes did not generate protective immunity (Figure 3). By contrast, only 5 µg of OVA encapsulated in FLs exhibited enhanced antitumor effects, as demonstrated by reduced tumor growth and prolonged survival. These findings suggested that FLs are efficient DNA vaccine carriers for the induction of antigen-specific immunity. Thus, by overcoming the current problems in genetic immunization, the FL-mediated gene transfer system represents a significant progress towards the development of genetic cancer or viral vaccines [10,11].

FL-mediated cytoplasmic delivery of nanoparticles for the development of a cytosolic controlled gene-release system

Novel gene-based therapies using AODNs and siRNAs represent a promising approach to decrease or modulate the expression of target molecules [44–46]. Since the main physiological target of these drugs is mRNA, it is pivotal to deliver them into the cytoplasm. Although there are systems, including ours, that can achieve the delivery of soluble drugs into the cytoplasm [23], a novel delivery system to introduce NPs containing gene-based drugs, such as AODNs and siRNAs, into the cytoplasm will provide further advantages for maintenance of the optimal concentration by protecting the genes from hydrolytic and enzymatic degradation. In this context, we developed a cytosolic NP delivery system using FLs [11,14]. We first evaluated the encapsulation of NPs into FLs using fluorescein isothiocyanate (FITC)-labeled NPs (500 nm in diameter). FITC-labeled NP-containing FLs (FL/NP) were successfully separated from unreacted liposomes and Sendai virus by sucrose-density gradient centrifugation. Cryo-transmission electron microscopic (TEM) analysis indicated that these separated samples were identical to FL/NP because the lipid membranes in the fractions exhibited the spiked structure of the envelope proteins derived from Sendai virus (Figure 4A–D). *In vitro* transfection studies demonstrated that FL delivered the NPs more efficiently into mammalian cells than NP encapsulating liposomes (Lipo/NP) and NP alone. Furthermore, TEM analysis revealed that NPs existed in the

Figure 3. Antitumor effect of fusogenic liposomes containing antigen-expression plasmid-mediated vaccination.



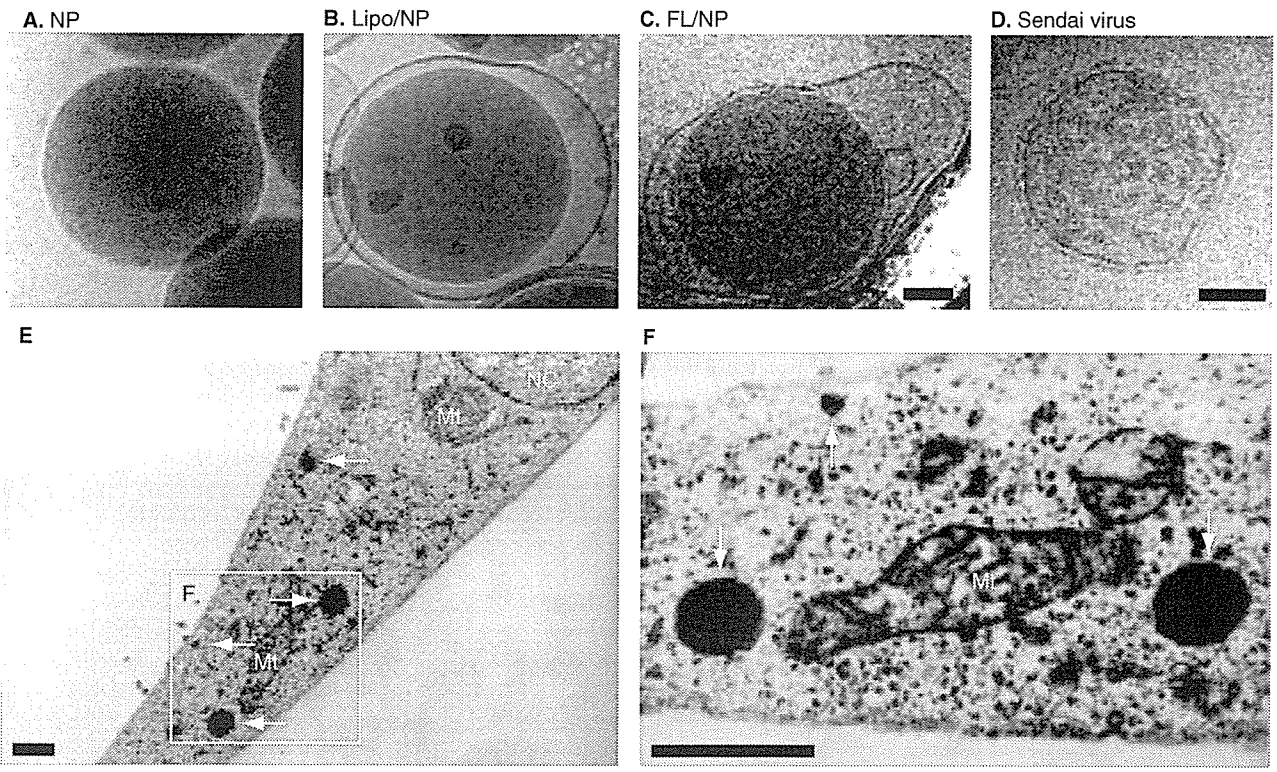
Mice were subcutaneously immunized twice at a 2-week interval with 5 µg ovalbumin (OVA) expression plasmid encapsulated in fusogenic liposomes **(A)**, conventional liposomes **(B)** or complexed with cationic liposomes **(C)**. The control group was immunized with 50 µg pOVA solution **(D)** or phosphate buffered saline **(E)**. A total of 4 weeks after the final immunization, immunized mice were challenged with 10^6 E.G7-OVA cells. The tumor size was measured at indicated time points, and the individual tumor size was graphed.

cytoplasm, not in the endosomes, of cells treated with FL/NP (Figure 4E & F). Consistent with the TEM analysis, FLs delivered NPs into mammalian cells in the presence of various endocytosis inhibitors (e.g., cytochalasin B, sodium azide, 2,4-dinitrophenol, nocodazole and colchicine). These results clearly indicated that FL delivered encapsulated NPs into the cytosol via membrane fusion rather than by endocytosis. To demonstrate a potential application of the FL/NP system for gene delivery, we examined the delivery of DNA oligonucleotides by FL/NP. FITC-labeled phosphorothioate oligonucleotides were immobilized on surface cationized poly (vinyl amine) NPs and encapsulated into FLs. Similarly to the above observations, flow cytometry analysis demonstrated that the delivery efficiency of FL/NP was superior to that of Lipo/NP or NPs alone. We used a single type of NP for the gene delivery experiment. Progress in nanotechnology over the last decade has enabled us to select NPs exhibiting different drug release profiles based on their characteristics (e.g., surface electron characteristics, hydrophile- and lipophile-balance) [47,48]. Since FLs can encapsulate and deliver various kinds of NPs into cells, NPs with different drug release profiles can be introduced into a cell, which might produce a timed drug release. This system is now under investigation. We conclude that FLs are effective delivery vehicles to introduce encapsulated NPs into the cytoplasm, and that the combinatorial nanotechnology, FL/NP system, will provide an opportunity for kinetic regulation of genetic drugs in the cytoplasm, which lead to a prolongation of gene expression or knockdown period.

Conclusion

This review describes the feasibility and suitability of FLs as gene delivery vehicles. FLs can deliver macromolecules, such as nucleic acids, proteins

Figure 4. Characterization of fusogenic liposomes containing nanoparticles and analysis of fusogenic liposomes containing nanoparticle-treated mammalian cells by Cryo-transmission electron microscopic.



FL/NP were prepared by fusing ultraviolet-inactivated Sendai virus with Lipo/NP. The resultant was purified by stepwise sucrose-density centrifugation. The fractions identical to NP **(A)**, Lipo/NP **(B)**, FL/NP **(C)** or Sendai virus **(D)** were observed with Cryo-TEM. Bar = 100 nm. Cells were cultured with FL/NP for 30 min and observed with Cryo-TEM. Bars are 1 μm at ×7000 **(E)** or ×20000 **(F)** magnification. Arrowheads indicate NPs.
FL: Fusogenic liposomes; FL/NP: Fusogenic liposomes containing nanoparticles; Lipo/NP: Liposomes containing nanoparticles; Mt: Mitochondrion; NC: Nucleus; NP: Nanoparticles.

and peptides as well as NPs, across the plasma membrane and into living cells both *in vivo* and *in vitro*. These results clearly demonstrated that FLs are an effective and useful approach to analyze the gene function and develop gene therapy.

Future perspective

As a tool for gene regulation, FLs are a promising delivery vehicle that can efficiently translocate macromolecules into the cytoplasm and alter the expression of selective genes. Furthermore, if combined with nucleic acid-encapsulated and/or adsorbed NPs, this system can be used to regulate the intracellular nucleic acid release profile, which will contribute to the development of optimal regulated or therapeutic effects. We anticipate that in the future the FL-mediated cytosolic delivery system will be a promising tool in the field of gene regulation and gene therapy.

Executive summary

Characterization of fusogenic liposomes

- Fusogenic liposomes (FLs) are composed of ultraviolet-inactivated Sendai virus and conventional liposome, and displaying Sendai virus-derived accessory proteins (hemagglutinin–neuraminidase and fusion protein) on its surface. This unique hybrid-liposome, FL system, can deliver various macromolecules (plasmids and oligonucleotides) into cytoplasm by membrane fusion.

Feasibility of FLs as gene delivery vehicles

- In comparison with cationic liposomes, FL-mediated gene delivery is rapid and more efficient, even under high-serum concentration.
- FL-mediated effective gene transfer system possesses high potential as a tool for *in vivo* gene therapy.
- The author has succeeded in the preparation of nanoparticle encapsulating-FLs. This system could provide an opportunity for kinetic regulation of genetic drugs, such as antisense-oligonucleotides or short interfering RNA, in the cytoplasm.

Conclusion & future perspective

- FLs are a unique cytosolic delivery carrier, which can deliver various macromolecules such as plasmids, oligonucleotides or nanoparticles. These techniques will be a powerful tool in the field of gene regulation and gene therapy.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Lu PY, Xie F, Woodle MC: *In vivo* application of RNA interference: from functional genomics to therapeutics. *Adv. Genet.* 54, 117–142 (2005).
2. Zhang YC, Taylor MM, Samson WK, Phillips MI: Antisense inhibition: oligonucleotides, ribozymes, and siRNAs. *Methods Mol. Med.* 106, 11–34 (2005).
3. Lu PY, Xie FY, Woodle MC: siRNA-mediated antitumorigenesis for drug target validation and therapeutics. *Curr. Opin. Mol. Ther.* 5(3), 225–234 (2003).
4. Luo D, Saltzman WM: Synthetic DNA delivery systems. *Nat. Biotechnol.* 18(1), 33–37 (2000).
5. Wiethoff CM, Middaugh CR: Barriers to nonviral gene delivery. *J. Pharm. Sci.* 92(2), 203–217 (2003).
6. Nishikawa M, Huang L: Nonviral vectors in the new millennium: delivery barriers in gene transfer. *Hum. Gene Ther.* 12(8), 861–870 (2001).
7. Mizuguchi H, Nakanishi M, Nakanishi T, Nakagawa T, Nakagawa S, Mayumi T: Application of fusogenic liposomes containing fragment A of diphtheria toxin to cancer therapy. *Br. J. Cancer* 73(4), 472–476 (1996).
8. Nakanishi M, Mizuguchi H, Ashihara K *et al.*: Gene delivery systems using the Sendai virus. *Mol. Membr. Biol.* 16(1), 123–127 (1999).
9. Nakanishi M, Mizuguchia H, Ashihara K *et al.*: Gene transfer vectors based on Sendai virus. *J. Control Release* 54(1), 61–68 (1998).
- **Characterizes the fusogenic liposomes (FL) as gene delivery vehicles. These authors demonstrated that plasmids containing FL could deliver their contents into mammalian cells via membrane fusion.**
10. Yoshikawa T, Imazu S, Gao JQ *et al.*: Augmentation of antigen-specific immune responses using DNA-fusogenic liposome vaccine. *Biochem. Biophys. Res. Commun.* 325(2), 500–505 (2004).
11. Yoshikawa T, Tsutsumi Y, Nakagawa S: Development of nanomedicine using intracellular DDS. *Nippon Rinsho* 64(2), 247–252 (2006).
12. Yoshikawa T, Imazu S, Gao JQ *et al.*: Non-methylated CpG motif packaged into fusogenic liposomes enhance antigen-specific immunity in mice. *Biol. Pharm. Bull.* 29(1), 105–109 (2006).
13. Yoshikawa T, Okada N, Tsujino M *et al.*: Vaccine efficacy of fusogenic liposomes containing tumor cell-lysate against murine B16BL6 melanoma. *Biol. Pharm. Bull.* 29(1), 100–104 (2006).
14. Kunisawa J, Masuda T, Katayama K *et al.*: Fusogenic liposome delivers encapsulated nanoparticles for cytosolic controlled gene release. *J. Control Release* 105(3), 344–353 (2005).
- **Our first report about cytosolic delivery of nanoparticles by FL. This study demonstrated that FL could deliver a large number of nanoparticles into cytoplasm, and this system could provide an opportunity for kinetic regulation of genetic drug.**
15. Sugita T, Yoshikawa T, Gao JQ *et al.*: Fusogenic liposome can be used as an effective vaccine carrier for peptide vaccination to induce cytotoxic T lymphocyte (CTL) response. *Biol. Pharm. Bull.* 28(1), 192–193 (2005).
16. Nakanishi T, Hayashi A, Kunisawa J *et al.*: Fusogenic liposomes efficiently deliver exogenous antigen through the cytoplasm into the MHC class I processing pathway. *Eur. J. Immunol.* 30(6), 1740–1747 (2000).
17. Markwell MA, Paulson JC: Sendai virus utilizes specific sialyloligosaccharides as host cell receptor determinants. *Proc. Natl Acad. Sci. USA* 77(10), 5693–5697 (1980).
18. Hosaka Y: Sendai virion structure and its interaction with cellular membranes. *Electron Microsc. Rev.* 1(1), 17–38 (1988).
19. Ishida N, Homma M: Sendai virus. *Adv. Virus Res.* 23, 349–383 (1978).

20. Uchida T, Kim J, Yamaizumi M, Miyake Y, Okada Y: Reconstitution of lipid vesicles associated with HVJ (Sendai virus) sikes. Purification and some properties of vesicles containing nontoxic fragment A of diphtheria toxin. *J. Cell Biol.* 80(1), 10–20 (1979).

• **First article to demonstrate that hybrid liposome consisting of Sendai virus and liposome could deliver its contents into cytoplasm. Mutant diphtheria toxin fragment A-containing lipid vesicles (liposomes) with Sendai virus spikes bound to mamalian cell membrane and released their fragment A into the cytoplasm causing cell death.**

21. Nakanishi M, Uchida T, Sugawa H, Ishiura M, Okada Y: Efficient introduction of contents of liposomes into cells using HVJ (Sendai virus). *Exp. Cell Res.* 159(2), 399–409 (1985).
22. Mizuguchi H, Nakanishi T, Kondoh M *et al.*: Fusion of Sendai virus with liposome depends on only F protein, but not HN protein. *Virus Res.* 59(2), 191–201 (1999).
23. Kunisawa J, Nakagawa S, Mayumi T: Pharmacotherapy by intracellular delivery of drugs using fusogenic liposomes: application to vaccine development. *Adv. Drug Deliv. Rev.* 52(3), 177–186 (2001).
24. Mizuguchi H, Nakagawa T, Nakanishi M, Imazu S, Nakagawa S, Mayumi T: Efficient gene transfer into mammalian cells using fusogenic liposome. *Biochem. Biophys. Res. Commun.* 218(1), 402–407 (1996).
25. Mizuguchi H, Nakagawa T, Toyosawa S *et al.*: Tumor necrosis factor α-mediated tumor regression by the *in vivo* transfer of genes into the artery that leads to tumors. *Cancer Res.* 58(24), 5725–5730 (1998).
26. Shiver JW, Davies ME, Perry HC, Freed DC, Liu MA: Humoral and cellular immunities elicited by HIV-1 vaccination. *J. Pharm. Sci.* 85(12), 1317–1324 (1996).
27. Webster RG, Fynan EF, Santoro JC, Robinson H: Protection of ferrets against influenza challenge with a DNA vaccine to the haemagglutinin. *Vaccine* 12(16), 1495–1498 (1994).
28. Donnelly JJ, Friedman A, Martinez D *et al.*: Preclinical efficacy of a prototype DNA vaccine: enhanced protection against antigenic drift in influenza virus. *Nat. Med.* 1(6), 583–587 (1995).
29. Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL: DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl Acad. Sci. USA* 90(24), 11478–11482 (1993).
30. Wang R, Doolan DL, Le TP *et al.*: Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 282(5388), 476–480 (1998).
31. Zhang L, Widera G, Bleecher S, Zaharoff DA, Mossop B, Rabussay D: Accelerated immune response to DNA vaccines. *DNA Cell Biol.* 22(12), 815–822 (2003).
32. Cohen AD, Boyer JD, Weiner DB: Modulating the immune response to genetic immunization. *Faseb J.* 12(15), 1611–1626 (1998).
33. Scheerlinck JY: Genetic adjuvants for DNA vaccines. *Vaccine* 19(17–19), 2647–2656 (2001).
34. Kawabata K, Takakura Y, Hashida M: The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. *Pharm. Res.* 12(6), 825–830 (1995).
35. Parker SE, Vahlsing HL, Serfilippi LM *et al.*: Cancer gene therapy using plasmid DNA: safety evaluation in rodents and non-human primates. *Hum. Gene Ther.* 6(5), 575–590 (1995).
36. Lew D, Parker SE, Latimer T *et al.*: Cancer gene therapy using plasmid DNA: pharmacokinetic study of DNA following injection in mice. *Hum. Gene Ther.* 6(5), 553–564 (1995).
37. Cui Z, Mumper RJ: The effect of co-administration of adjuvants with a nanoparticle-based genetic vaccine delivery system on the resulting immune responses. *Eur. J. Pharm. Biopharm.* 55(1), 11–18 (2003).
38. Gregoriadis G, Saffie R, de Souza JB: Liposome-mediated DNA vaccination. *FEBS Lett.* 402(2–3), 107–110 (1997).
39. Locher CP, Putnam D, Langer R, Witt SA, Ashlock BM, Levy JA: Enhancement of a human immunodeficiency virus env DNA vaccine using a novel polycationic nanoparticle formulation. *Immunol. Lett.* 90(2–3), 67–70 (2003).
40. Mumper RJ, Cui Z: Genetic immunization by jet injection of targeted pDNA-coated nanoparticles. *Methods* 31(3), 255–262 (2003).
41. Cui Z, Hsu CH, Mumper RJ: Physical characterization and macrophage cell uptake of mannan-coated nanoparticles. *Drug Dev. Ind. Pharm.* 29(6), 689–700 (2003).
42. Cui Z, Baizer L, Mumper RJ: Intradermal immunization with novel plasmid DNA coated nanoparticles via a needle-free injection device. *J. Biotechnol.* 102(2), 105–115 (2003).
43. Cui Z, Mumper RJ: Intranasal administration of plasmid DNA-coated nanoparticles results in enhanced immune responses. *J. Pharm. Pharmacol.* 54(9), 1195–1203 (2002).
44. Opalinska JB, Gewirtz AM: Nucleic-acid therapeutics: basic principles and recent applications. *Nat. Rev. Drug Discov.* 1(7), 503–514 (2002).
45. Suwanmanee T, Sierakowska H, Fucharoen S, Kole R: Repair of a splicing defect in erythroid cells from patients with β-thalassemia/HbE disorder. *Mol. Ther.* 6(6), 718–726 (2002).
46. Song E, Lee SK, Wang J *et al.*: RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* 9(3), 347–351 (2003).
47. Moghimi SM, Hunter AC, Murray JC: Nanomedicine: current status and future prospects. *Faseb J.* 19(3), 311–330 (2005).
48. LaVan DA, McGuire T, Langer R: Small-scale systems for *in vivo* drug delivery. *Nat. Biotechnol.* 21(10), 1184–1191 (2003).

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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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