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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·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

高建青[1*]，陈海靓[1]，中川晋作[2]，水口裕之[3]，梁文权[1]

(1. 浙江大学 药物制剂研究所，浙江 杭州 310031; 2. 日本大阪大学 药学研究科，日本 大阪 565-0871; 3. 日本国立生物医学创新研究所，日本 大阪 567-0085)

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

GAO Jian-qing[1*], CHEN Hai-liang[1], NAKAGAWA Shinsaku[2], MIZUGUCHI Hiroyuki[3], LIANG Wen-quan[1]

(1. Institute of Pharmaceutics, Zhejiang University, Hangzhou 310031, China; 2. Graduate School of Pharmaceutical Sciences, Osaka University, suita 565-0871, Japan; 3. National Institute of Biomedical Innovation, Osaka 567-0085, Japan)

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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*通讯作者　Tel: 86 - 571 - 87217128, Fax: 86 - 571 - 87217376, E-mail: gaojianqing1029@yahoo.com.cn

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2×10^3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1×10^6个接种于6～8周龄的BALB/c小鼠皮下,一周后将2×10^8 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·

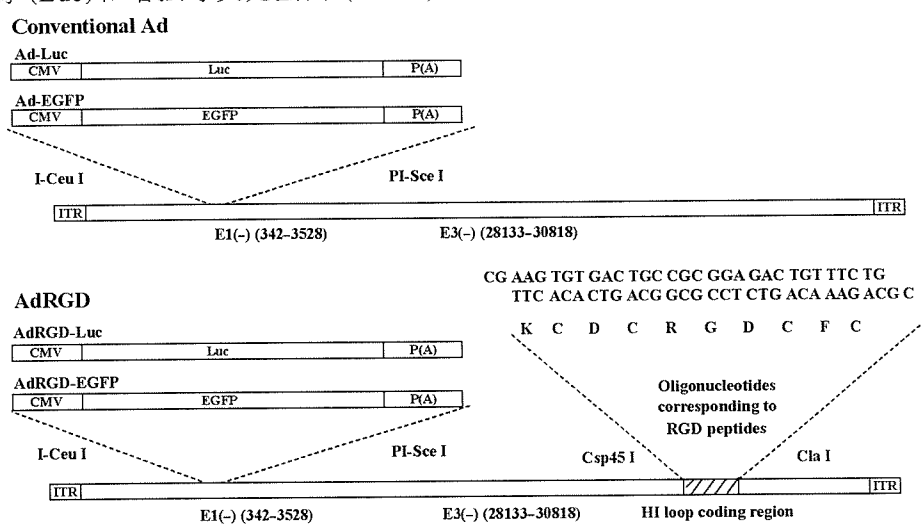

Figure 1 Representative of the construction of recombinant adenovirus vectors

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。

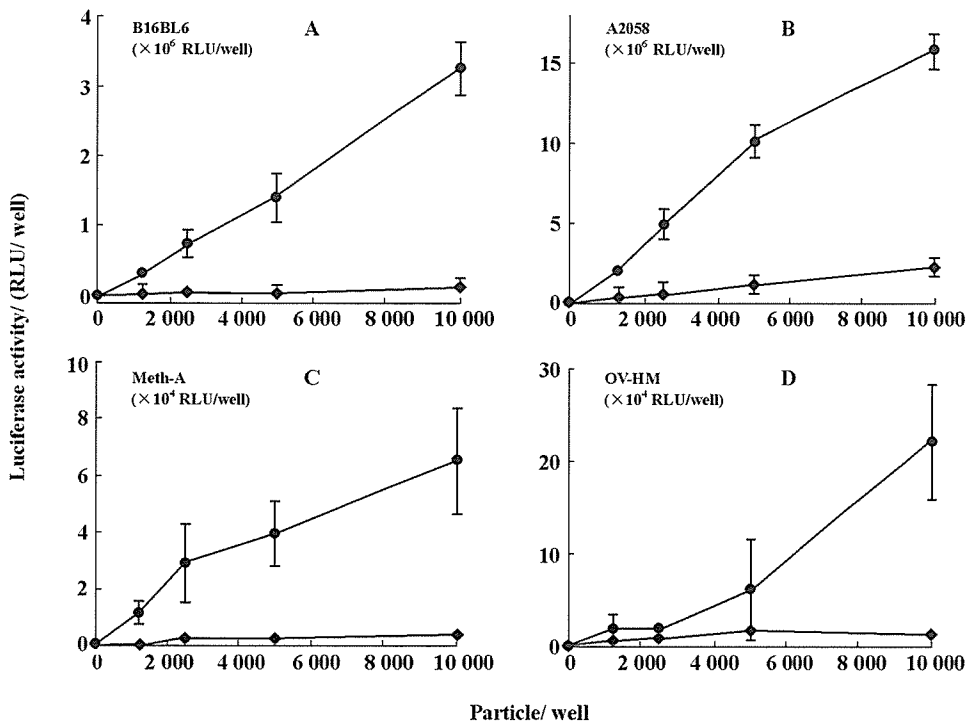

Figure 2 Luciferase expression of Ad-Luc or AdRGD-Luc transfected tumor cells 2×10^3 of B16BL6 melanoma cells, A2058 melanoma cells, OV-HM ovarian carcinoma cells, and Meth-A fibrosarcoma cells per well in 96-well plate were transfected with Ad-Luc (◆) or AdRGD-Luc (●) separately at indicated viral particles/cell 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

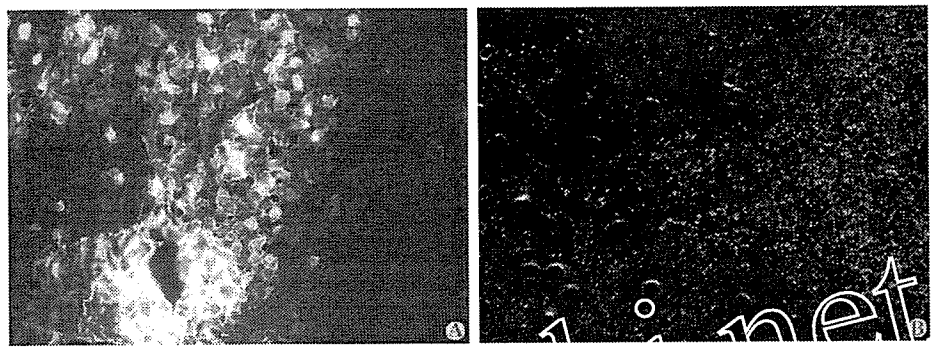

Figure 3 Gene expression in Meth-A tumor via intratumoral injection. 1 $\times 10^6$ Meth-A cells were inoculated intradermally and after 1 week, (A) 2 $\times 10^8$ pfu/tumor AdRGD-EGFP or (B) 2 $\times 10^8$ pfu/tumor Ad-EGFP were intratumoral injected. Tumor nodes were harvested and sectioned using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

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。

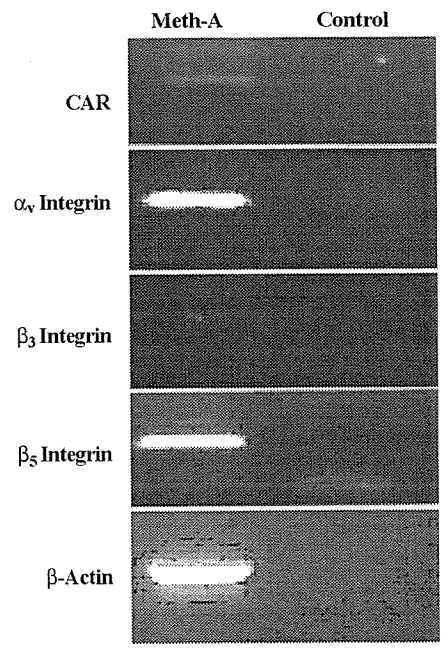

Figure 4 RT-PCR analysis of CAR, α_v-integrin, β_3-integrin, and β_5-integrin in Meth-A fibrosarcoma. Total RNA was prepared from Meth-A fibrosarcoma cells and then RT-PCR was performed as described in Table 1

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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REVIEW

Fusogenic liposomes and their suitability for gene delivery

Tomoaki Yoshikawa, Naoki Okada & Shinsaku Nakagawa†

†Author for correspondence Osaka University, Department of Biotechnology & Therapeutics, Graduate School of Pharmaceutical Sciences, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
Tel.: +81 06 6879 8176; Fax: +81 06 6879 8176; nakagawa@phs.osaka-u.ac.jp

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

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.

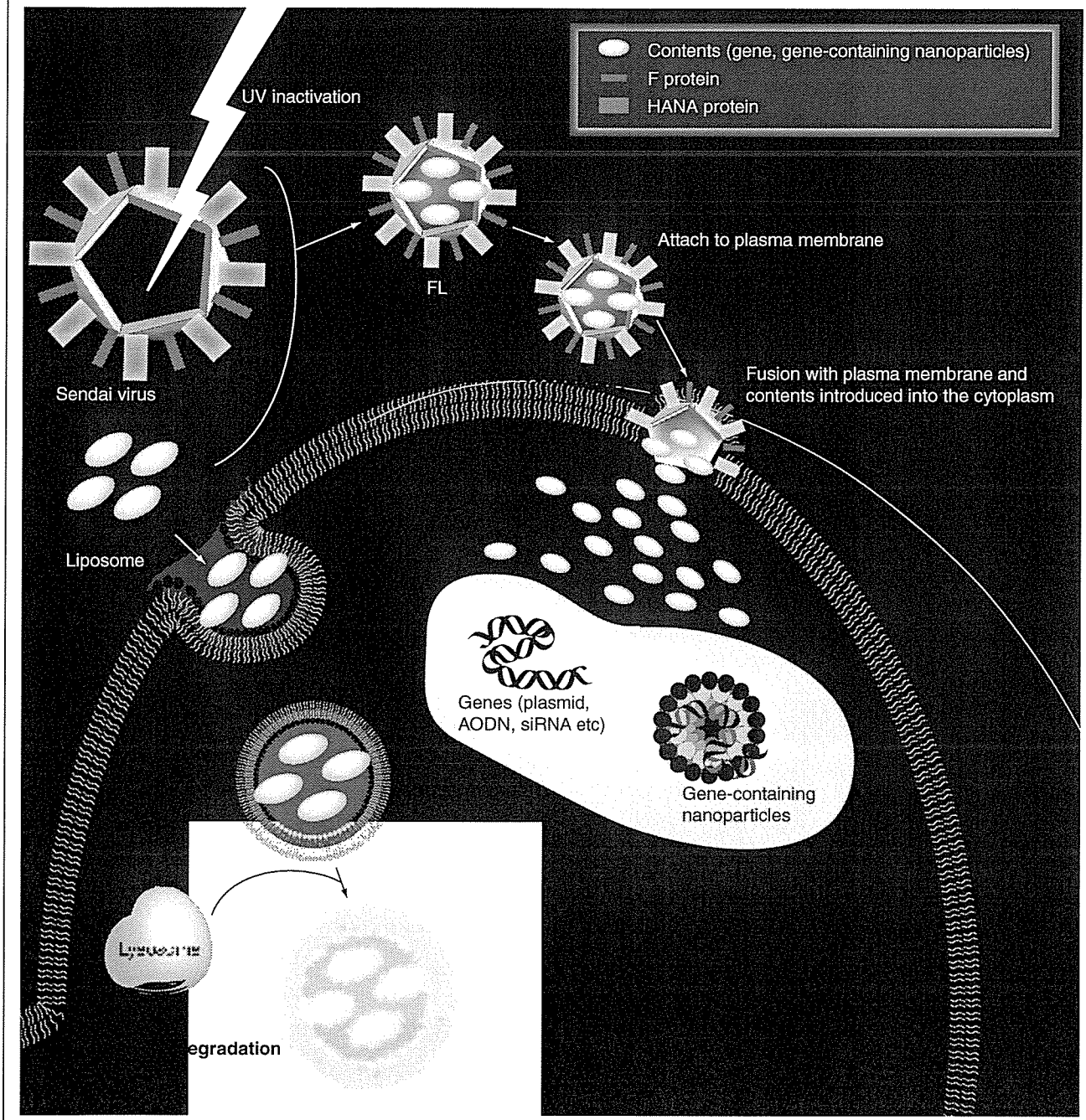
We developed a hybrid delivery system utilizing fusogenic liposomes (FLs), which are composed of conventional liposomes and Sendai virus. Previous studies indicated that FLs deliver encapsulated contents into the cytosol efficiently, rapidly and directly [7–15]. Early reports demonstrate that membrane 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

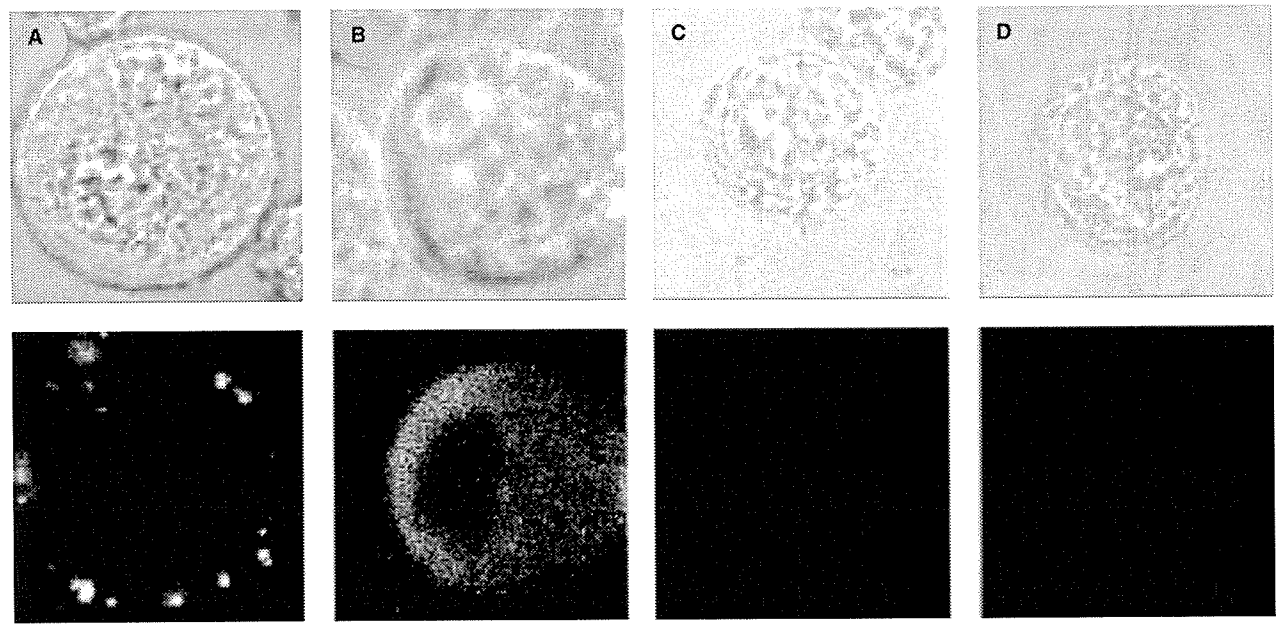
liposomes [20,21]. In addition, they reported that F proteins, but not HANA proteins, are essential for the fusion step between liposomes and Sendai virus [22]. From these reports, the Sendai virus receptor (sialic acid) is not required for the fusion step because sialic acid does not exist on liposomes. 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

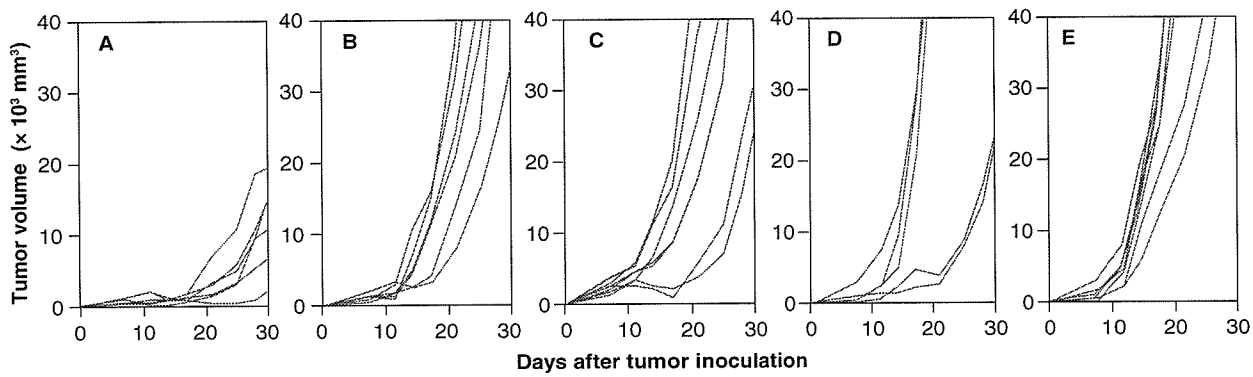
S-180 tumor cells were intraperitoneally injected into male ddY mice at day 0. At 5 days, luciferase expression plasmids encapsulated in fusogenic liposomes or complexed with cationic liposomes were given intraperitoneally. At 7 days after injection, S-180 cells were recovered and luciferase activity 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.



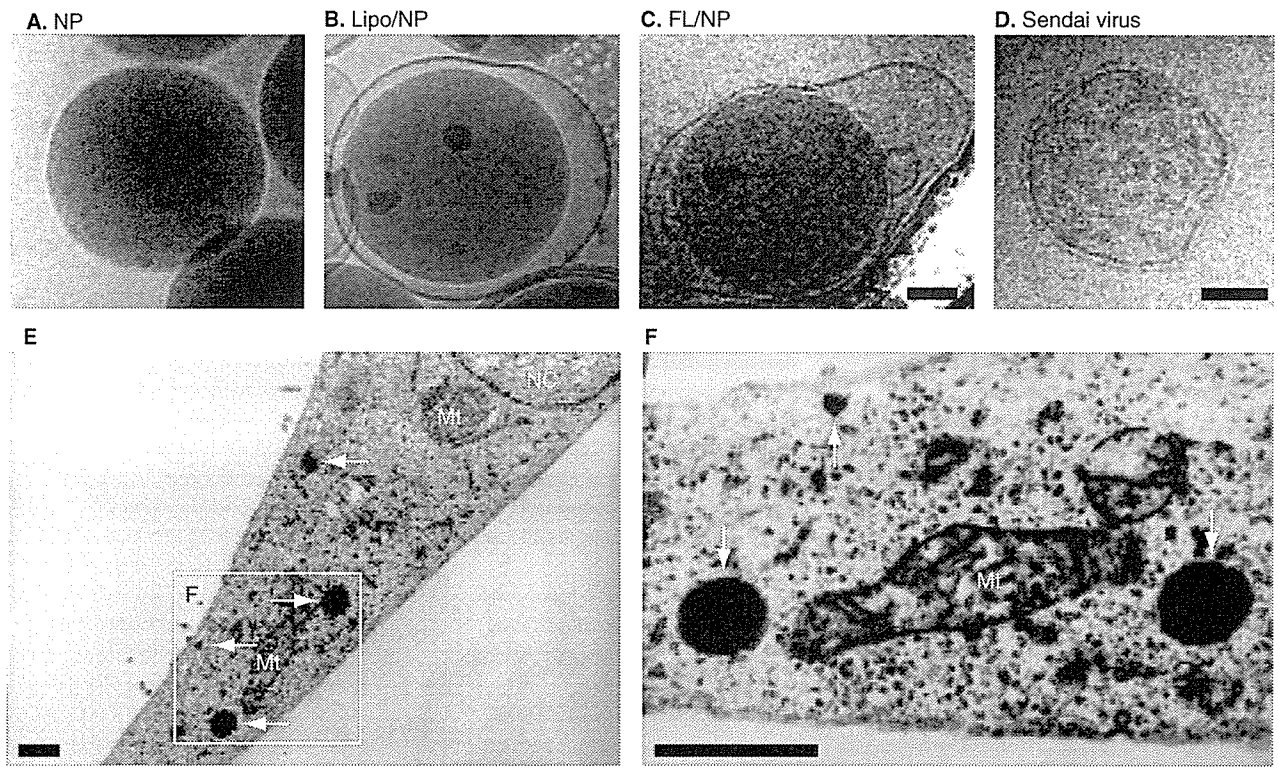
Mice were subcutaneously immunized twice at a 2-week interval with 5 µg ovalbumin (OVA) expression plasmid encapsulated in fusogenic liposomes **(A)**, conventional liposomes **(B)** or complexed with cationic liposomes **(C)**. The control group was immunized with 50 µg pOVA solution **(D)** or phosphate 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.

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Affiliations

- *Tomoaki Yoshikawa*
  Osaka University, Department of Biotechnology & Therapeutics, Graduate School of Pharmaceutical Sciences, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
  Tel.: +81 06 6879 8177;
  Fax: +81 06 6879 8177;
  tomoaki@phs.osaka-u.ac.jp
- *Naoki Okada*
  Osaka University, Department of Biotechnology & Therapeutics, Graduate School of Pharmaceutical Sciences, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan,
  and,
  Core Research for Evolutional Science & Technology (CREST), Japan Science & Technology Agency (JST), Tokyo, Japan
  Tel.: +81 06 6879 8178;
  Fax: +81 06 6879 8178;
  okada@phs.osaka-u.ac.jp
- *Shinsaku Nakagawa*
  Osaka University, Department of Biotechnology & Therapeutics, Graduate School of Pharmaceutical Sciences, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
  Tel.: +81 06 6879 8175;
  Fax: +81 06 6879 8175;
  nakagawa@phs.osaka-u.ac.jp

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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

Fuminori Sakurai [a], Sayaka Murakami [a,b], Kenji Kawabata [a], Naoki Okada [c], Akira Yamamoto [b], Tsukasa Seya [d], Takao Hayakawa [e], Hiroyuki Mizuguchi [a,c,*]

[a] *Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka, 567-0085, Japan*
[b] *Department of Biopharmaceutics, Kyoto Pharmaceutical University, Kyoto, 607-8414, Japan*
[c] *Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, 565-0871, Japan*
[d] *Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Sapporo, 060-8638, Japan*
[e] *Pharmaceuticals and Medical Devices Agency, Tokyo, 100-0013, Japan*

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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

* Corresponding author. Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, 7-6-8 Asagi, Saito, Ibaragi-City, Osaka, 567-0085, Japan. Tel.: +81 72 641 9815; fax: +81 72 641 9816.
E-mail address: mizuguch@nibio.go.jp (H. Mizuguchi).

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

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

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

2. Materials and methods

2.1. Cells and antibodies

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

2.2. Plasmids

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

2.3. Flowcytometric analysis of CD46 expression

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

Table 1
Monoclonal anti-CD46 antibodies used in this study

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

2.4. Adenovirus vectors

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

2.5. Transduction experiments

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

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

2.6. Real-time quantitative PCR

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

3. Results

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

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

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

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

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

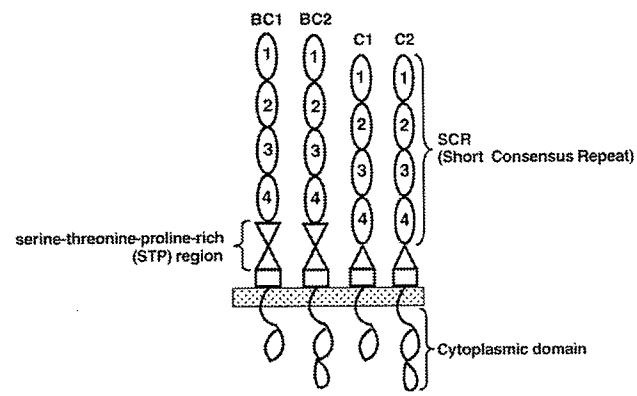

Fig. 1. A schematic diagram of human CD46. Human CD46 is ubiquitously expressed in almost all human cells mainly as four isoforms (BC1, BC2, C1, C2) that are derived via alternative splicing. Human CD46 is composed of four cysteine-rich short consensus repeats (SCRs), a serine–threonine–proline-rich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

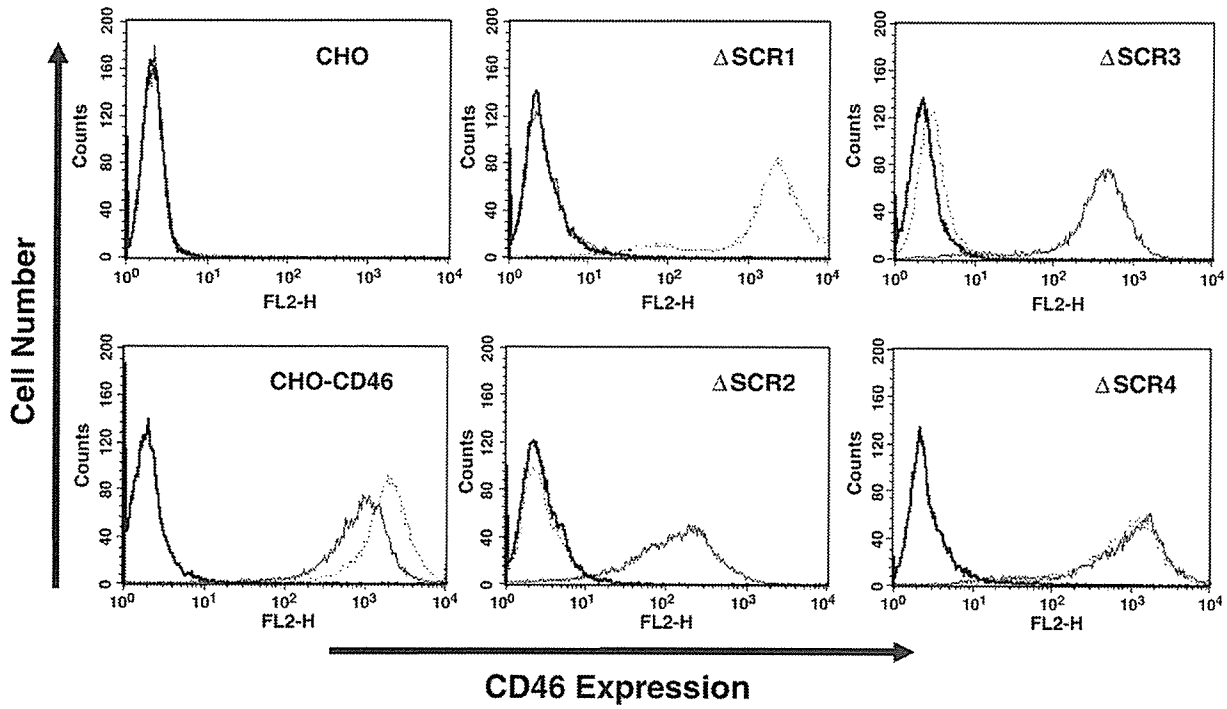

Fig. 2. Expression profiles of CD46 deletion mutants detected by monoclonal anti-CD46 antibodies. The cells were stained with anti-CD46 antibodies against SCR1 (E4.3; thin line), or SCR2 (M177; dotted line), followed by a PE-labeled secondary antibody, and subsequently analyzed by a flowcytometer. ΔSCR3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

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

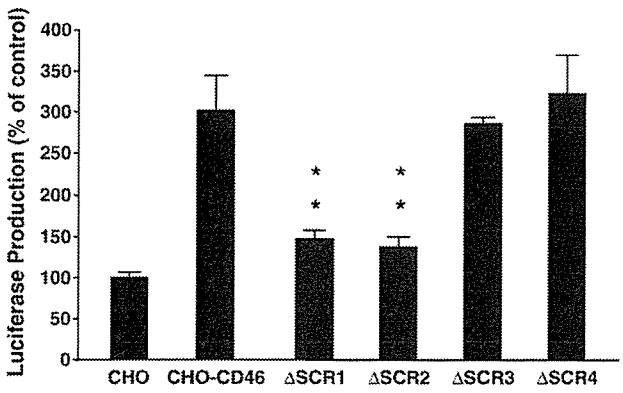

Fig. 3. Ad35L-mediated transduction in CHO cells expressing CD46 mutants lacking SCRs. The cells were transduced with Ad35L at 3000 VP/cell for 1.5 h. The luciferase productions in the cells were measured 48 h after transduction by luminescent assay. The data were normalized to the luciferase production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

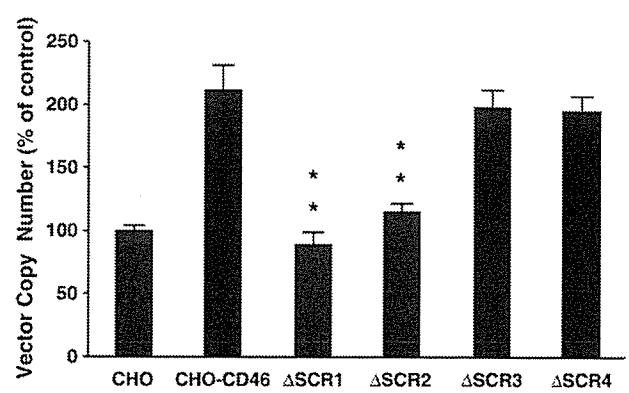

Fig. 4. Cellular uptake of Ad35L in CHO cells expressing CD46 mutants lacking SCRs. The cells were transduced with Ad35L at 3000 VP/cell for 1.5 h. The total DNA, including the vector DNA, was extracted from the cells 48 h after transduction. The copy numbers of the vector DNA were quantified by TaqMan-PCR.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

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

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

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

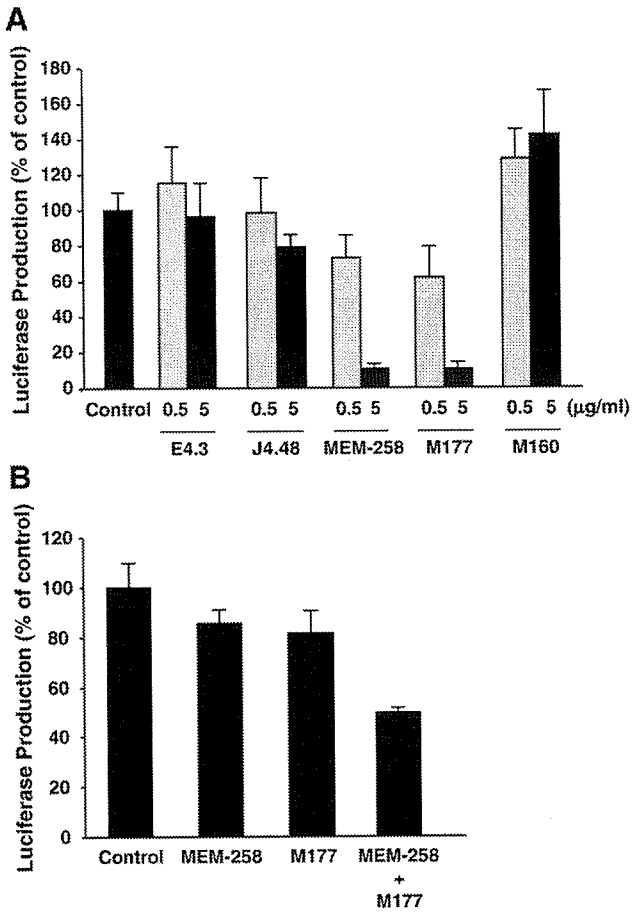

Fig. 5. Blocking of Ad35L-mediated transduction by monoclonal anti-CD46 antibodies. (A) Inhibition of Ad35L-mediated transduction by monoclonal anti-CD46 antibodies. E4.3, MEM-258, and J4-48 (recognizing SCR1), M177 (recognizing SCR2), and M160 (recognizing SCR3) were used as monoclonal anti-CD46 antibodies.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

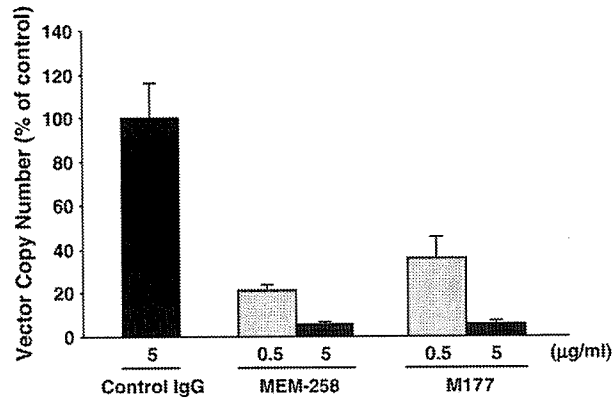

Fig. 6. Inhibition of cellular uptake of Ad35L by monoclonal anti-CD46 antibodies. CHO transformants expressing full-length CD46 were transduced with Ad35L in the presence of anti-CD46 antibody MEM-258 and M177 as described in Fig. 5. The total DNA, including the vector DNA, was extracted 48 h after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

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

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

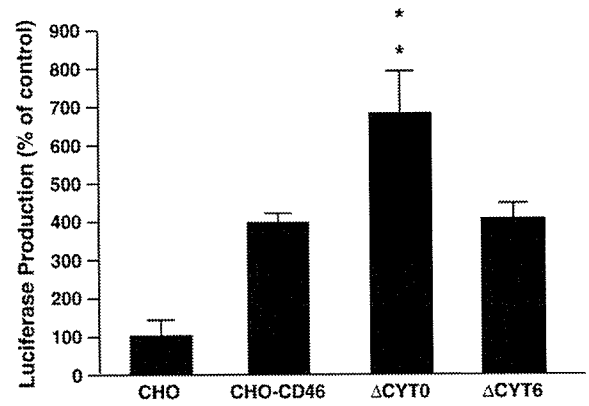

Fig. 7. Ad35L-mediated transduction in CHO cells expressing CD46 mutants lacking the cytoplasmic domain. The cells were transduced with Ad35L at 3000 VP/cells for 1.5 h. The luciferase productions in the cells were measured 48 h after transduction by luminescent assay. The data were normalized to the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