Adenovirus vectors with chimeric type 5 and 35 fiber proteins exhibit enhanced transfection of human pancreatic cancer cells

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Abstract. Adenovirus (Ad) vectors are widely used for gene transfer. Efficient gene transfer into malignant cells is an important requirement for anticancer gene therapy, but transgene expression after transfer with adenoviral vectors varies among different cancer cell lines. Recently, Ad vectors containing chimeric type 5 and 35 fiber proteins have been developed. We evaluated the expression of coxsackie and adenovirus receptor (CAR), as well as integrins αV, ß3 and ß5, in seven human pancreatic cancer cell lines and assessed the relationship between expression of these molecules and Ad transfection efficiency. We compared the transfection efficiency of a conventional type 5 Ad vector (Ad5GFP) with that of an Ad vector containing chimeric type 5 and 35 fiber proteins (Ad5/35GFP), which expressed green fluorescent protein (GFP) driven by the cytomegalovirus promoter. There was strong CAR expression by AsPC-1, CFPAC-1 and PANC-1 cells, whereas the other cell lines showed weak expression. There was strong integrin ß3 expression by MIAPaCa-2, PANC-1 and Suit-2 cells, but expression by AsPC-1, BxPC-3, CFPAC-1 and HPAC cells was weak. Transfection efficiency of the vectors for human pancreatic cancer cell lines was not directly related to the CAR or integrin expression. However, transfection by Ad5/35GFP was significantly greater than by Ad5GFP at MOIs of 10 and 25 in all five human pancreatic cell lines. In conclusion, the Ad5/35GFP vector mediates more efficient gene transfer to human pancreatic cancer cells. These results may have implications for improving the efficiency of Ad-mediated gene transfer and developing adenoviral vectors.

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Key words: pancreatic neoplasms, conditionally replicative adenovirus vectors, fiber chimeric Ad5/35

Introduction

Adenovirus (Ad) has a linear double-stranded DNA genome of ~35,000 base pairs. The DNA is packaged in an icosahedral capsid protein, which is 70-100 nm in diameter. Each virion (viral particle) consists of a DNA core surrounded by a protein shell composed of 252 subunits called capsomeres, 240 of which are hexons and 12 of which are pentons. A rod-like structure called a fiber protrudes from the base of each penton (1,2). At least 51 serotypes of human Ad have been identified and classified into six distinct subgroups (A-F).

Among them, type 2 and type 5 (which belong to subgroup C) have been the most extensively studied and type 5 (Ad5) has been widely used as a vector for gene therapy because of its ability to infect a wide variety of cells, propagate at a high titer *in vitro* and support efficient expression of transgenes.

Infection of susceptible cells by Ad involves two distinct steps. In the first step, high-affinity binding of the virus to its primary receptor on the cell surface, the coxsackie and adenovirus receptor (CAR), occurs via the C-terminal knob domain of the fiber protein (3-5). In the second step, interaction between the RGD (Arg-Gly-Asp) motifs of the penton bases and secondary host cell receptors (αVß3 and αVß5 integrins) facilitates internalization of the virus via receptor-mediated endocytosis (6-8). Therefore, deficiency of CAR and/or αVß3 and αVß5 integrins will limit the gene transfer capacity of Ad5-based vectors.

Ad serotype 35 (Ad35), which belongs to subgroup B, has primarily been isolated from the kidneys and lungs of renal transplant patients (9). Ad35 has shorter fiber proteins (7 repeats of the ß sheet) than Ad5 (22 repeats of the ß sheet) and shows a different pattern of tropism from that of Ad5. CD46 is a receptor for Ad35 that is ubiquitously expressed in human cells (10,11). Ad vectors containing chimeric type 5 and 35 fiber protein have been developed (12,13). The Ad5/35 vector can transduce CAR-negative cell lines and various human cell lines more effectively than the Ad5

vector. Therefore, the Ad5/35 vector could be a promising candidate for efficient gene transfer to human cancer cell lines.

In the present study, to evaluate the possibility of developing gene therapy for pancreatic cancer, we examined the expression of CAR, as well as αV, ß3 and ß5 integrins, by human pancreatic cancer cell lines. Then we evaluated the ability of the Ad5/35 vector to transfer genes to these human pancreatic cell lines in comparison with that of the Ad5 vector.

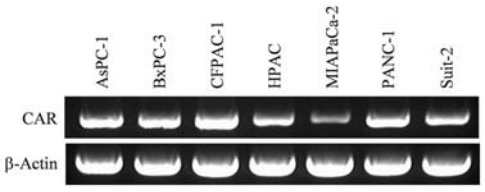

Figure 1. Expression of CAR mRNA by human pancreatic cancer cells. CAR mRNA was detected in all cell lines. ß-Actin was used as the internal control.

Materials and methods

Cell culture. Seven human pancreatic cancer cell lines were used. AsPC-1, BxPC-3, CFPAC-1, HPAC, MIAPaCa-2 and PANC-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in the medium recommended by the ATCC at 37˚C under a humidified atmosphere of 5% CO_2. Suit-2 cells were kindly provided by Dr H. Tomoda (National Kyushu Cancer Center, Fukuoka, Japan) and were cultured in DMEM (Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (FBS) (ICN Biomedicals, Aurora, OH). Human embryonic kidney (HEK) 293 cells were purchased from RIKEN Bioresourse Center (Tukuba, Japan). Each medium contained 100 U/ml of penicillin and 0.1 mg/ml streptomycin (Gibco-BRL).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA was isolated using TRIzol Reagent (Life Technologies, Rockville, MD). Then cDNA was prepared by random priming from 1 μg of total RNA using a first-strand cDNA synthesis kit (Pharmacia Biotech, North Peapack, NJ) according to the manufacturer's instruction. Five microliters of first-strand cDNA solution was subjected to the polymerase chain reaction (PCR) with synthetic oligonucleotide primers (Nippon EGT, Toyama, Japan). The sequences of the primers used in this study were as follows: CAR, forward: 5'-GCAGGAGCCTTATAGGAA CTTTG-3', reverse: 5'-GGACCCCAGGGATGAATGAT-3' Integrin αV forward: 5'-GAAGATGTTGGGCCAGTTGT-3', reverse: 5'-TCCACAACCCAAAGTGTGAA-3', Integrin ß5 forward: 5'-AGCACCAAGAGAGATTGCGT-3', reverse: 5'-GGTGTTTCCACACTCTGGCT-3', ß-actin, forward: 5'-GGCATCGTGATGGACTCCG-3', reverse: 5'-GCTGGA AGGTGGACAGCGA-3'. The products were 195, 381, 246 and 613 base pairs in size, respectively.

The reproducibility of the technique and quality of the total RNA was confirmed by using ß-actin primers. Sequences were amplified by 30 PCR cycles (30 sec of denaturation at 94˚C, 30 sec of annealing at 57˚C and 1 min of extension at 72˚C), followed by final extension at 72˚C for 10 min. Then the PCR products were analyzed on 2% agarose gel.

Quantitative RT-PCR. To monitor CAR gene expression, we performed quantitative real-time RT-PCR analysis based on the Taq Man fluorescence method. This method uses a dual-labeled non-extendable oligonucleotide hydrolysis (Taq Man) probe in addition to the two amplification primers. The probe contains 6-carboxy-fluorescein (FAM) as a fluorescent reporter dye, and 6-carboxytetramethyl-rhodamine (TAMRA) as a quencher for light emission. During the extension phase of PCR, the probe hybridizes to the target sequence and is then cleaved by the 5' to 3' exonuclease activity of Taq polymerase. The increase in the fluorescence signal of the reporter is proportional to the amount of the specific PCR product, providing highly accurate and reproducible quantification. The increase of reporter dye emission is detected by an automated sequence detector combined with analysis software (ABI Prizm 7700 Sequence Detection System; PE Applied Biosystems, Foster City, CA). The reaction conditions were set according to the manufacturer's protocol. The following primers and Taq Man probe were used for analysis. The specific primers for CAR were: 5'-CGACTGCAAGTACAA GTTTGAGAAC-3' (forward primer), 5'-TCTCCTGGCACT GAGCATTG-3' (reverse primer), 5' (FAM)-AAGGCACCC TGAAGAAGGCGCG-(TAMRA) 3' (Taq Man probe). The cycle parameters were heating at 95˚C for 10 min (for activation of Taq-Polymerase), followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Detection of ß-actin RNA for assessment of quality and normalization was done with the Taq Man ß-actin Control Reagent kit (PE Applied Biosystems), which utilizes standard Taq Man probe chemistry.

Immunocytochemistry. Pancreatic cancer cells grown on glass slides were washed with TBS, then fixed with 4% paraformaldehyde in TBS for 10 min, washed again with TBS, permeabilized in 0.2% Triton X-100 in TBS for 5 min and washed and blocked with TBS containing 1% BSA. Fixed and permeabilized cells were incubated with a monoclonal anti-CAR RmcB antibody (#05-644, Upstate Biotechnology, Lake Placid, NY) for 1 h at room temperature, rinsed twice with TBS and then incubated for 30 min with the secondary antibody (goat anti-mouse IgG) (#62-6500, Zymed Laboratories, South San Francisco, CA) at room temperature. Cells were washed 3 times with TBS and incubated for 20 min with Cy3-conjugated donkey anti-goat IgG antibody (#Ap180C, Chemicon International, Temecula, CA). After the final wash, a coverslip was mounted on each slide with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Then the cells were examined under a fluorescene microscope (Olympus, Tokyo, Japan).

Flow cytometry. Cells were briefly trypsinized, washed with PBS and incubated for 1 h at 4˚C with primary antibodies targeting CAR and integrin ß3 in PBS containing 2% FBS. Mouse monoclonal anti-CAR antibody was used at a dilution of 1:100. Then the cells were washed with PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG (#A1102, Molecular Probes, Eugene, OR) at 1:1,000 for 45 min at 4˚C. Cells were washed again with PBS and analyzed by flow

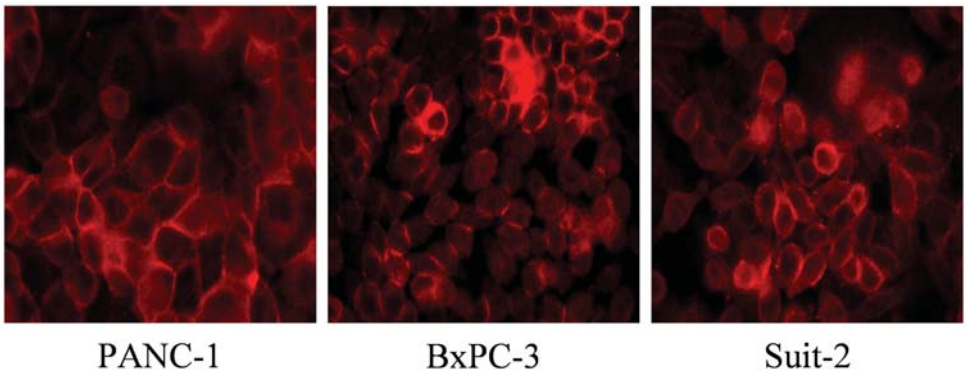

Figure 2. Membrane expression of CAR on pancreatic cancer cells. Cells were grown on glass slides, fixed with methanol and immunostained with an anti-CAR monoclonal antibody (RmcB) and a Cy3-conjugated donkey anti-mouse antibody. Confocal images of the pancreatic cancer cell lines PANC-1, BxPC-3 and Suit-2 are shown.

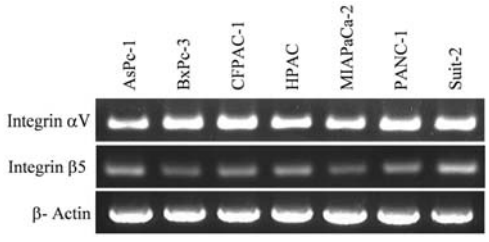

Figure 3. Expression profile of integrin αV and ß5 mRNA in human pancreatic cancer cell lines. ß-Actin was used as the internal control.

Table I. Relative mRNA levels of CAR in human pancreatic cancer cells.

Cell lines	CAR mRNA/ß-actin mRNA (mean ± SE)
AsPC-1	0.130±0.017
BxPC-3	0.100±0.009
CFPAC-1	0.087±0.004
HPAC	0.027±0.003
MIAPaCa-2	0.012±0.001
PANC-1	0.071±0.003
Suit-2	0.023±0.001

cytometry on a FACSCalibur using Cell Quest software (Becton-Dickinson, Franklin, NJ).

Adenovirus-mediated gene transduction of human pancreatic cancer cells. A type 5, E1A-deleted, replication-defective Ad vector containing the green fluorescent protein gene (Ad5GFP) was constructed with the AdEasy XL Adenoviral Vector System (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The Ad5/35 GFP vector was purchased from Zymed Laboratories Inc. Each Ad was propagated in HEK 293 cells, purified by two rounds of cesium chloride density centrifugation, dialyzed and stored at -70˚C. Viral titers were determined with an Adeno-X Rapid Titer kit (BD Biosciences Clontech, Palo Alto, CA).

Transfection efficiency for human pancreatic cancer cells was compared between the Ad5GFP and Ad5/35 GFP vectors. Cancer cells were transfected with Ad5GFP or Ad5/35 GFP at various multiplicities of infection (MOI) for 16 to 18 h after seeding. At 48 h after transfection, GFP expression in the cells was determined by FACS analysis.

Statistical analysis. Results are presented as the mean ± SEM for quantitative experiments and each experiment was performed at least three times independently. To compare GFP expression by flow cytometry, the t-test was used. Statistical analysis was done with JMP statistical software and significance was considered to be present at $p<0.05$.

Results

Expression of CAR and integrins by pancreatic cancer cell lines. Expression of CAR mRNA by the human pancreatic cancer cell lines is shown in Fig. 1. This mRNA was detected in all of the cell lines tested by RT-PCR. The CAR mRNA/ß-actin mRNA expression ratio is shown in Table I for each cell line.

Next, we performed immunocytochemistry and flow cytometry to determine the surface expression of CAR by the cells lines, because CAR is a membrane-associated protein. Immunofluorescence was observed on the cell membrane and not in the cytoplasm. Fig. 2 shows typical membrane expression of CAR by AsPC-1, CFPAC-1 and PANC-1 cells.

We also examined the expression of integrins αV and ß5 by human pancreatic cancer cells. The mRNA of integrins αV and ß5 was expressed in all cell lines tested (Fig. 3).

The relative expression of CAR and integrin ß3 was measured by flow cytometry. There was strong expression of CAR by AsPC-1, CFPAC-1 and PANC-1 cells, while expression by BxPC-3, HPAC and Suit-1 and MIAPaCa-2 cells was weak (Fig. 4). Protein expression was identical to mRNA expression for each cell line. There was strong integrin ß3 expression by MIAPaCa-2, PANC-1 and Suit-2 cells, while expression by AsPC-1, BxPC-3, CFPAC-1 and HPAC cells was weak (Fig. 5). The mean fluorescence intensity (MFI) of CAR and integrin ß3 expression is summarized in Table II.

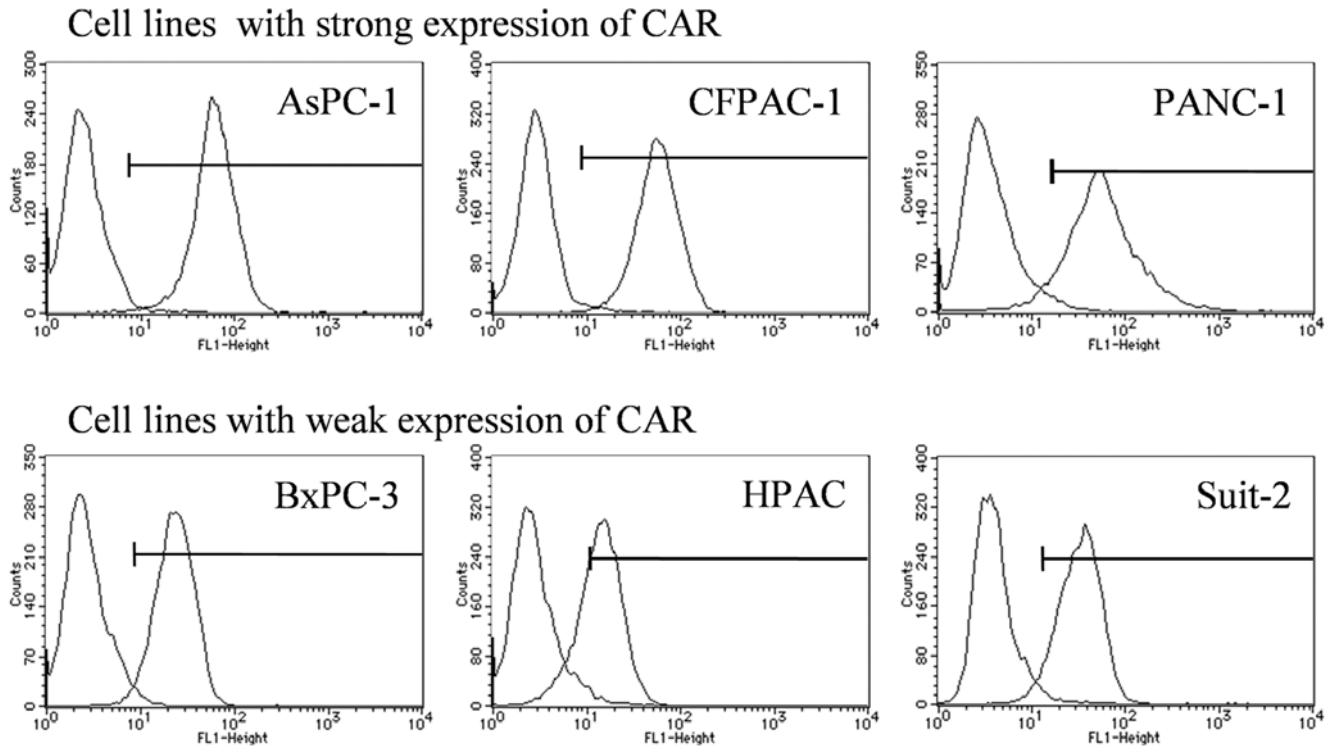

Figure 4. Flow cytometric analysis of the cell membrane expression of CAR protein after immunostaining with RmcB (anti-CAR antibody). AsPC-1, CFPAC-1 and PANC-1 cells show strong expression of CAR (upper row), while BxPC-3, HPAC and Suit-1 show weak expression (lower row).

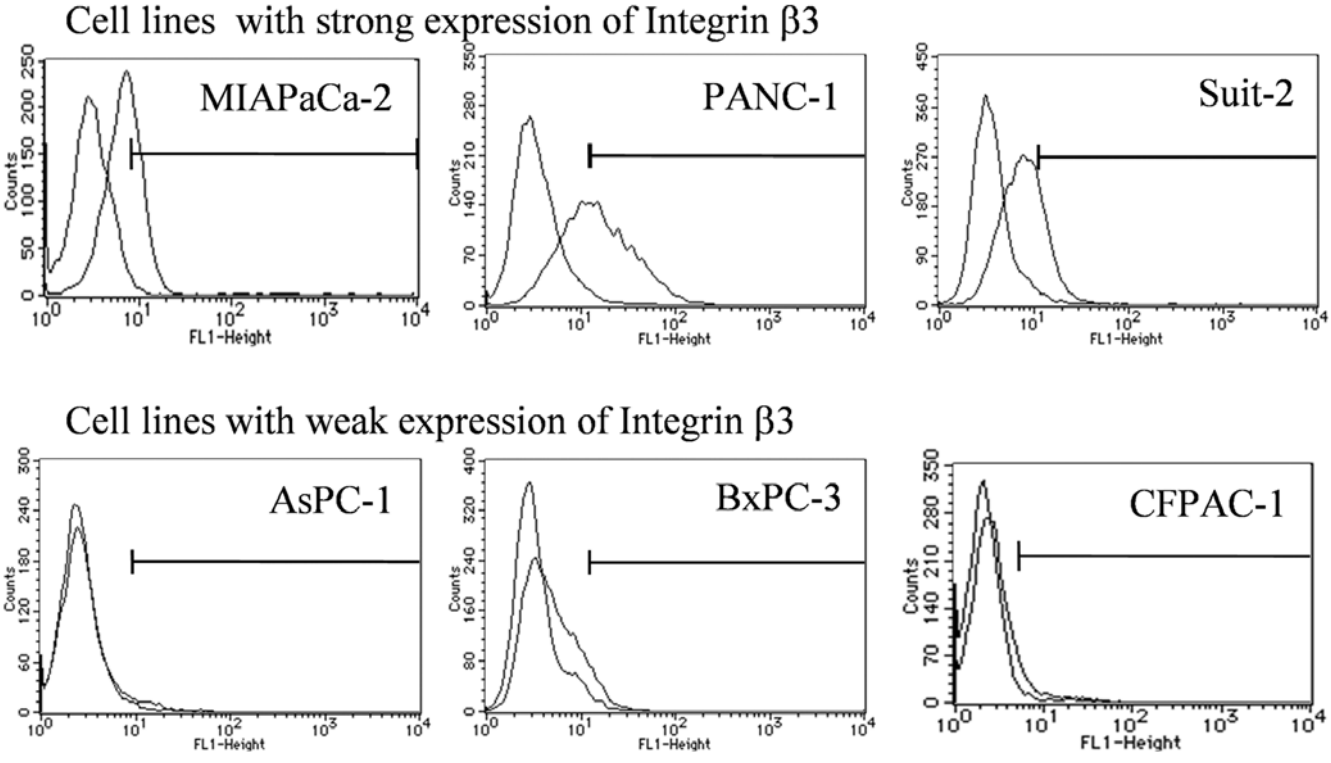

Figure 5. Flow cytometric analysis of the expression of integrin ß3 by human pancreatic cancer cell lines after staining with a primary anti-integrin ß3 monoclonal antibody followed by a fluorescein-conjugated goat anti-mouse secondary antibody. MIAPaCa-2, PANC-1 and Suit-2 cells show strong expression of integrin ß3 (upper row), while AsPC-1, BxPC-3 and CFPAC-1 cells show weak expression (lower row).

Sensitivity of human pancreatic cancer cell lines to infection with adenovirus type 5 and chimeric type 5/35. The induction efficacy of Ad5GFP vector into human pancreatic cancer cells was examined. Cancer cells were infected with Ad5GFP at a MOI of 1, 10, 25, 50 and 100 at 16 to 18 h after seeding. After 48 h, GFP-expressing cells were detected by flow cytometry.

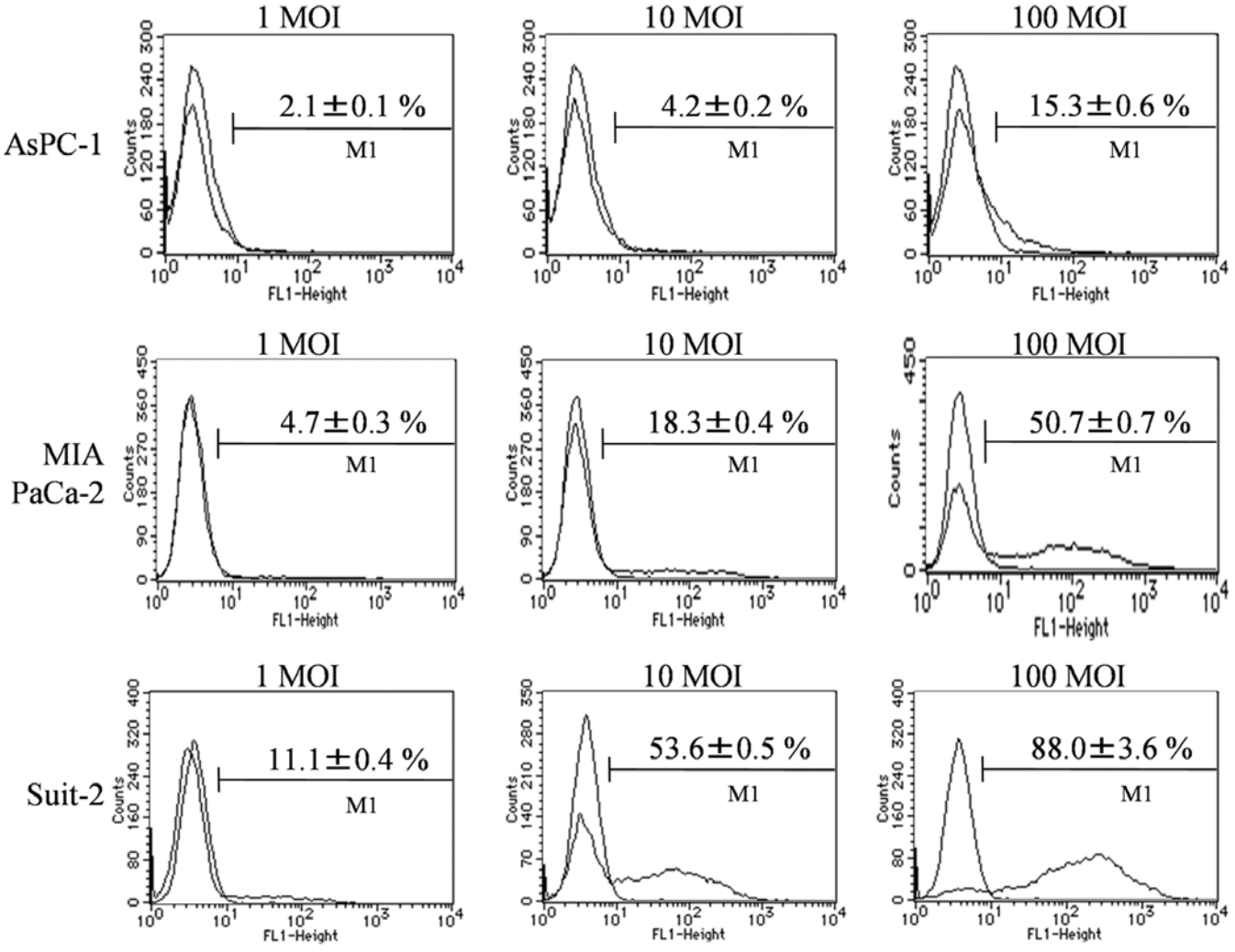

Figure 6. Human pancreatic cancer cells were seeded into a 6-cm plate and the Ad5GFP vector (1, 10, or 100 MOI) was added at 16 to 18 h after seeding. After 48 h, GFP expression was measured by flow cytometry. Data represent the mean ± SE of three experiments.

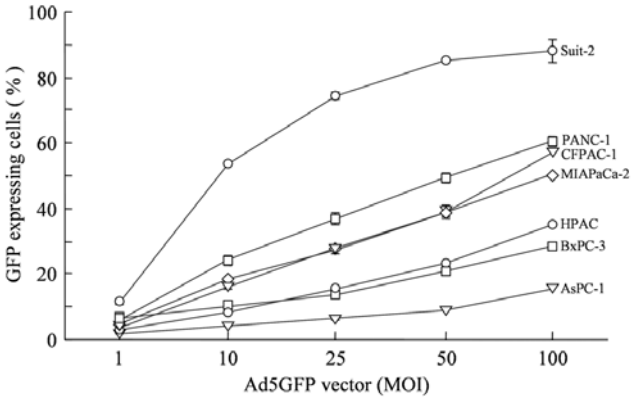

Figure 7. MOI-dependent increase of GFP expression by human pancreatic cancer cells infected with Ad5GFP at various MOIs. In each cell line, GFP expression increased in a MOI-dependent manner. Data are the mean ± SE of three experiments.

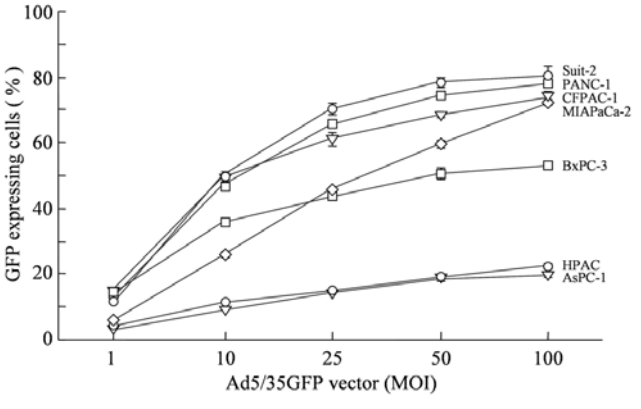

Figure 8. MOI-dependent increase of GFP expression by human pancreatic cancer cells infected with Ad5/35GFP at various MOIs. In each cell line, GFP expression increased in a MOI-dependent manner. Data are the mean ± SE of three experiments.

The relative mean fluorescence intensity increased in a MOI-dependent manner. As shown in Fig. 6, GFP expression driven by the Ad5GFP vector in AsPC-1, MIAPaCa-2 and Suit-1 cells increased as the MOI increased.

The transfection efficiency of the Ad5 and Ad5/35 vectors was also cell-specific. GFP expression at 48 h after infection by Ad5GFP and Ad5/35GFP is summarized in Figs. 7 and 8. Both vectors induced a dose-dependent increase of GP

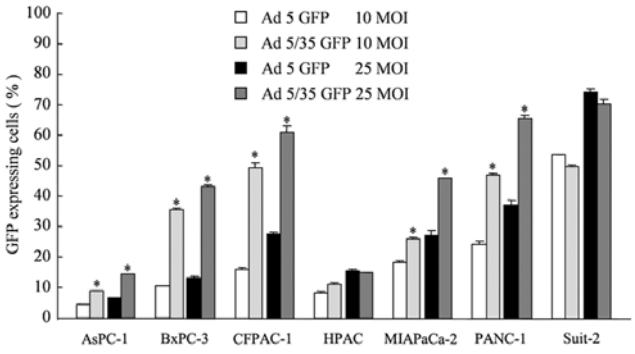

Figure 9. Comparison of the transfection efficiency of the conventional Ad5 vector Ad5GFP and chimeric Ad5/35GFP vector in human pancreatic cancer cell lines. Data are the mean ± SE of three experiments. Asterisks indicate a significant difference compared with the corresponding MOI of Ad5GFP.

Table II. The cell surface expression of CAR and integrin ß3 in human pancreatic cancer cell lines.

Cell lines	Mean fluorescence intensity (MFI)	
	CAR	Integrin ß3
AsPC-1	23.3±0.8	1.03±0.02
BxPC-3	8.5±0.1	1.05±0.01
CFPAC-1	23.6±0.4	1.12±0.02
HPAC	4.9±0.1	1.14±0.01
MIAPaCa-2	3.8±0.1	2.13±0.08
PANC-1	15.4±0.1	4.25±0.09
Suit-2	7.7±0.1	2.10±0.02

expression by all cell lines. However, at the same MOI (e.g. 10 or 25), the percentage of GFP-positive cells was significantly higher with the chimeric type Ad5/35GFP vector than the Ad5GFP vector, except in the case of HPAC and Suit-2 cells (Fig. 9).

Discussion

It has been reported that two cell surface events, attachment and internalization, are required for Ad to enter host cells (7). First, the viral fiber protein attaches to the CAR on the surface of a host cell (3) and then the virus enters the cell through interaction of its penton base with integrins αVß3 and αVß5 on the host cell membrane (7). Therefore, the expression of these cell surface proteins and their correlation with viral transfection efficiency have been investigated and it has been reported that integrins αVß3 (14,15), αVß5 (16,17) and αVß1 (18) are important for efficient gene transfer as well as CAR (19,20). Similarly, Qin *et al* (21) have reported that CAR expression *in vitro* is correlated with susceptibility to Ad infection.

Pearson *et al* (22) have reported that a positive association between the level of integrin ß3 expression and efficiency of Ad gene transfer. They postulated that although CAR and integrins αVß5, αV and ß1 are all required for the entrance of adenoviral vectors into host cells, these proteins may not be the limiting factor once a minimum level of expression is present. Apart from the expression patterns of these molecules, it has been stated that characterization of molecules having a known association with viral entry into cells is important to improve adenovirus-mediated gene transfer.

Ad vectors containing chimeric type 5 and 35 fiber protein have been developed (12,13). The Ad5/35 vector can transduce CAR-negative cell lines and various human cell lines more effectively than the Ad5 vector, so this vector could be a promising candidate for efficient gene transfer to human cancer cell lines. The group B serotype Ad35 virus recognizes a different receptor from CAR (23), which has been identified as CD46 (10,24), a molecule that is ubiquitously expressed by human cells (11). The Ad5/35 chimeric vector is identical with the Ad5 parent vector, except for the difference of fiber construction. Because both vectors used in this study contained a GFP reporter transgene driven by the cytomegalovirus promoter, differences of transfection efficiency and transgene expression should be related to the influence on the viral vector of this change in the fiber protein.

In this study, we evaluated the transfection efficiency of Ad5 and Ad5/35 using several human pancreatic cancer cell lines and found that the chimeric Ad5/35 vector could transfect the cell lines more efficiently than the conventional Ad5 vector. For example, at 25 MOI, 43.5% of BxPC-3 cells expressed GFP after transfection with Ad5/35GFP, while the conventional Ad5GFP vector only achieved GFP expression in 13.5% of BxPC-3 cells. There were only two cell lines (HPAC and Suit-2) for which the transfection efficiency was equal between Ad5 and Ad5/35.

Improvement of transfection efficiency by chimeric vectors such as Ad5/35 has also been reported for other cells. After modification of the viral fiber (such as Ad5/3 or Ad5/35), better transfection efficiency is seen for ovarian cancer cells and hematopoietic stem cells compared with that of the parent Ad5 vector (25,26). These chimeric vectors bind to other receptors than CAR and enter the cell via an integrin-independent pathway (23,27). The Ad5/35 chimeric vector has been shown to infect both CAR-positive and CAR-negative hematopoietic stem cells efficiently and to mediate high levels of transgene expression (28).

As expected, we found no direct relationship between Ad transfection efficiency and expression of CAR or integrin ß3. Interestingly, the vectors had low efficiency for AsPC-1 cells with almost no integrin ß3 expression. In conclusion, the Ad5/35 chimeric vector shows better transfection efficiency for human pancreatic cancer cells than that of Ad5 independent of the cell surface expression of molecules such as CAR and integrins. Modification of the viral fiber protein significantly improves the susceptibility of some pancreatic cancer cell lines that were not targeted by the conventional Ad5 vector. The enhancement of transgene expression that was achieved by infecting previously refractory cells suggests that modified fiber vectors may be useful for further studies of gene delivery to cancer cells.

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