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The Dual Impact of Coxsackie and Adenovirus Receptor Expression on Human Prostate Cancer Gene Therapy[1]

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Abstract

In a recent paper, we reported a significant difference in coxsackie and adenovirus receptor (CAR) from several human bladder cancer cell lines that correlated with their sensitivities to adenoviral infection (Y. Li, R-C. Pong, J. M. Bergelson, M, C. Hall, A. I. Sagalowsky, C-P. Tseng, Z. Wang, and J. T. Hsieh, Cancer Res., *59:* 325–330, 1999). In human prostate cancer, CAR protein is down-regulated in the highly tumorigenic PC3 cell line, which suggests that, in addition to its function as a viral receptor, CAR may have a pathophysiological role in prostate cancer progression. In this paper, we document that CAR does not merely enhance the viral sensitivity of prostate cancer cells but also acts as a tumor inhibitor for androgen-independent prostate cancer cells. Our data indicate that CAR is a potential therapeutic agent for increasing the efficacy of prostate cancer therapy.

Introduction

Adenovirus is a nonenveloped DNA virus thought to enter the host cell cytoplasm through a specific receptor-mediated endocytosis. Virus adsorption occurs when viral fibers, elongated proteins that project radically from each of 12 vertices of an icosahedral capsid, bind to specific cellular receptors on a target cell membrane (1). Recently, two groups (2, 3) reported cloning a unique transmembrane protein for both coxsackie and CAR.[4] Adenovirus type 5 is frequently used as a vector for gene therapy. Sequence analysis indicates that this CAR cDNA encodes a typical immunoglobulin-like membrane protein with two immunoglobulin domains that interact with adenovirus fiber protein (2, 3). In addition to the extracellular domain, CAR cDNA contains a 22-amino acid transmembrane domain and a 107-amino acid intracellular domain that has a putative tyrosine phosphorylation site. According to its protein structure, CAR may function not only as the receptor for adenovirus but also as a cell adhesion molecule. However, the physiological function of CAR is virtually unknown.

In the past 5 years, many studies (4–6) have explored adenovirus-based gene therapy on prostate cancer treatment. However, some key issues related to the efficiency of virus uptake or possible side effects have not been addressed. For example, recombinant adenoviruses have proved to be relatively inefficient in airway epithelia because they bind more poorly to the differentiated ciliated airway epithelia than to immature airway cells (7). On the other hand, viral proteins are good immunogens. High dosages of adenovirus may impose a potential host immune rejection. Obviously, increasing the susceptibility of target cells to viral infection increases the efficacy of gene therapy.

In our laboratory, we use replication-deficient adenovirus to evaluate the efficacy of gene therapy for prostate cancer. Previously, we reported that CAR expression varies among human bladder cancer cells, and we demonstrated that increased levels of CAR significantly enhance the uptake of adenovirus (8). In this study, we document that the levels of CAR protein expression among three human prostate cancer cell lines correlate with their *in vivo* tumorigenic potentials. This prompts us to examine the effect of increased CAR expression on the efficacy of prostate cancer therapy. Furthermore, because CAR is down-regulated in prostate cancer cells, we decided to examine the functional role of CAR in the growth of prostate cancer. The significance of these finding is discussed.

Materials and Methods

The PC3 cell lines used in this study were obtained from American Type Culture Collection (Manassas, VA) and were grown in T medium (9) containing 5% FBS. A mammalian expression vector, pcDNA3.1/V5/His-TOPO, was purchased from Invitrogen (Carlsbad, CA). Two replication-deficient recombinant viruses, AdCMV-β-gal and AdCMV-p21, were generated as described previously (2, 10).

Plasmid Construction and Transfection into Prostate Cancer Cells. We performed RT-PCR to obtain CAR cDNA, with total cellular RNA isolated from both 253J and RT4 cell lines, and CAR cDNA was assembled as described previously (9). Two additional CAR mutants (Tailess and GPI) were used in this study. Tailess CAR is a mutant with a deletion of the cytoplasmic domain of CAR cDNA (11). GPI cDNA, containing only the extracellular domain of CAR, was constructed by deleting both transmembrane and cytoplasmic domains and then adding a glycolipid anchor domain for membrane attachment as described previously (11). PC3 cells (2×10^5 per p-35 plate) were transfected with 2 μg of each plasmid using Lipofectamine transfection reagent. For selection of stable sublines, 48 h after transfection, cells were split and selected for neomycin-resistant clones with G-418 (600 μg/ml). Resistant colonies were either pooled or cloned by ring isolation after 2 weeks of selection.

Determination of CAR Levels by FACS. Cytometric analysis was used to determine CAR levels for each cell. Briefly, membrane fluorescence staining was performed on a single-cell suspension with the use of RmcB monoclonal antibody (11) and FITC-conjugated secondary antibodies as described previously (12). FACS was performed with a dual-laser Vantage flow cytometer (Becton Dickinson, Mountain View, CA) which delivered 50 mW at 488 nm with an Enterprise air-cooled laser. Analysis was performed using LYSYS II software (Becton Dickinson). The positive population of cells was determined by gating the right-hand tail of the distribution of the negative control sample for each individual cell line at 1%. This setting was then used to determine the percentage of positive cells for each of the above markers for each individual cell line.

Detection of Virus-mediated Gene Delivery. To determine the viral sensitivity of human prostate cancer cells, 5×10^5 cells were infected with different concentrations of AdCMV-β-gal at 37°C in a 5% CO-humidified incubator. Twenty-four hours after infection, the β-galactosidase activity (13)

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[4] The abbreviations used are: CAR, coxsackie and adenovirus receptor; FACS, fluorescent-activated cell scanning; m.o.i, multiplicity of infection; EGFR, epidermal growth factor receptor.

was measured in a 200 μl of cell lysate and normalized to the protein concentration of each sample.

Effect of P21 Adenovirus on the Growth Rate of CAR-expressing Prostate Cancer Cells *in Vitro*. The p21 adenovirus was used to determine the efficacy of gene therapy on the CAR-transfected PC3 sublines. Cells were plated at a density of 5000 cells in 48-well plates using T medium containing 0.2% FBS and infected with AdCMV-p21 at 0, 1, 10, and 100 m.o.i. At the indicated time, cells were harvested, and relative cell number was determined by crystal violet assay (14).

Western Analysis of p21 and pRb Expression. To examine the levels of p21 and Rb expression in PC3 cells after their infection with the p21 virus, we conducted a Western assay as described previously (10). The cell lysate was made by adding 20% SDS containing 1 mM phenylmethylsulfonyl fluoride. The lysate was sonicated for 30 s on ice, followed by centrifugation for 5 min at 4°C. From each sample, 20 μg of total protein were electrophoresed on a 10% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane. After the membrane was blocked with PBS containing 5% powdered milk, it was incubated with anti-p21 (6B6; PharMingen, San Diego, CA) or anti-Rb (G3-245; PharMingen) antibody for 1 h, followed by incubation with antimouse IgG. After extensive washing, the protein was visualized with an ECL chemiluminescence detection kit (Amersham, Arlington Heights, IL).

Determination of Both *in Vitro* and *in Vivo* Growth Rate of CAR-transfected PC3 Sublines. To examine the effect of CAR on the growth rate of cells, we measured the *in vitro* growth rate of CAR-transfected PC3 cells. Cells were plated at density of 5,000 cells in 48-well plates with T medium containing 0.2% FBS. Relative cell numbers were determined by crystal violet assay at the indicated time.

To determine the *in vivo* tumor growth of each transfected clone, we injected 1×10^6 cells/site at 6 sites s.c. in the flanks of 8- to 10-week-old male athymic mice. After 2 weeks inoculation, when tumors became palpable, growth of s.c. tumors was measured weekly with a caliper, and tumor volume was calculated (volume = length × width× height × 0.5236) (14).

Statistical Analysis. All data were evaluated by Student's test. Probabilities <0.05 were considered significant.

Results

Correlation of CAR Levels and Viral Sensitivity of Human Prostate Cancer. Cytometric analysis of the immunofluorescence staining of three human prostate cancer cell lines (LNCaP, DU145, and PC3) indicates that DU145 contains the highest number of CAR-positive cells (82%). LNCaP contains 63% of CAR-positive cells, and PC3 contains the lowest numbers of CAR-positive cells (35%). The viral sensitivity of each cell line, determined by AdCMV-β-gal correlated with the percentages of CAR-positive cells (Table 1), suggests that CAR levels may determine the viral sensitivity of each cell line.

To examine whether the CAR protein is responsible for adenoviral infection in human prostate cancer cells, we constructed a mammalian CAR expression vector and transfected it into PC3 cells. After G-418 selection, four independent clones of CAR (PC3-CAR2, -7, -11, and -14), one vector-transfected clone (PC3-vector), and four independent clones of CAR mutants (PC3-Tailess4, PC3-Tailess5, PC3-GPI4, and PC3-GPI12) were selected, based on the different plasmid DNA integration pattern (Fig. 2*A*).

Table 1 *Determination of CAR levels by FACS and the efficiency of virus-mediated gene delivery in CAR-, GPI-, or Tailess-transfected PC3 cells*

		β-Galactosidase activity ($A_{405\ nm}/\mu g$)[b]		
Cell line	FACS (%)[a]	Control	10 m.o.i.	100 m.o.i.
DU145	82	0.98 ± 0.36	35.68 ± 3.54	225.67 ± 4.52
LNCaP	63	1.11 ± 0.87	22.24 ± 2.87	145.87 ± 5.61
PC3-parental	35	1.21 ± 0.32	10.74 ± 1.36	48.09 ± 2.35
PC3-vector	11	1.40 ± 0.54	3.06 ± 0.99	16.20 ± 0.88
PC3-CAR2	86	1.25 ± 0.24	35.96 ± 0.98	239.39 ± 8.02
PC3-CAR7	45	1.37 ± 0.59	19.22 ± 2.73	77.12 ± 0.25
PC3-CAR11	63	1.08 ± 0.49	23.42 ± 0.99	120.87 ± 1.90
PC3-CAR14	46	1.17 ± 0.54	13.25 ± 0.46	89.46 ± 4.70
PC3-GPI4	54	1.45 ± 0.12	23.40 ± 3.04	117.17 ± 8.67
PC3-GPI12	61	1.52 ± 0.28	27.62 ± 1.21	126.86 ± 2.21
PC3-Tailess4	80	1.67 ± 0.92	39.21 ± 3.98	264.67 ± 4.85
PC3-Tailess5	62	1.14 ± 0.83	30.88 ± 0.87	173.82 ± 5.32

[a] Cells were incubated with RmcB (CAR) before the addition of FITC-conjugated antimouse IgG secondary antibody. Data are calculated as described in "Materials and Methods" and presented as the percentage of cells gated positive.

[b] Each value was determined in triplicate from two separate experiments.

Immunofluorescence staining of the transfected PC3 cells with CAR monoclonal antibody was performed 1 month after G418 selection. Results (Table 1) show that variable CAR-positive cells, ranging from 45 to 86%, were detected among six different PC3 subclones. In contrast, the CAR-positive cells in PC3-vector were ~10%. Cytometric analysis revealed that, overall, the CAR-positive cell population was enriched ~3–8 times greater than PC3-vector cells. Furthermore, results obtained from β-galactosidase activity (after infecting each transfected PC3 with AdCMV-β-gal) indicate that β-galactosidase activity is proportional to the CAR levels from each clone. In particular, β-galactosidase activity in PC3-CAR2 cells infected with 100 m.o.i. AdCMV-β-gal was ~15 times higher than that in PC3-vector cells. For two CAR mutants (GPI and Tailess sublines), they were still sensitive to adenovirus; the β-galactosidase correlated with the levels of membrane CAR determined by cytometric analysis (Table 1), suggesting that CAR appears to be a more efficient receptor. Taken together, these data indicate that viral sensitivities of prostate cancer cell lines correlate with their CAR levels.

Efficacy of Gene Therapy in CAR-expressing Prostate Cancer Cells. Demonstrating the presence of CAR is one of key determinants of the efficacy of gene therapy. To do this, a recombinant adenovirus carrying p21 cDNA, a cyclic kinase inhibitor (10), was used. As shown in Fig. 1, the PC3-parental, vector and CAR2 cells did not show growth inhibition at day 6 in the presence of 1 m.o.i. AdCMV-p21. However, AdCMV-p21 at 10 m.o.i. can exhibit an 84% growth inhibition rate in PC3-CAR2 cells at day 6 (Fig. 1*C*), whereas the same concentration of AdCMV-p21 achieved only a 22% growth inhibition rate in PC3-parental cells at day 6 (Fig. 1*A*). More significantly, as shown in Fig. 1*C*, the growth inhibition elicited by AdCMV-p21 at both 10 and 100 m.o.i. was almost identical. This indicates that the increased expression of CAR protein in PC3 cells can reduce the concentration of p21 virus by as much as 10 times to achieve maximal growth inhibition. Western blot analyses (Fig. 1*D*) provided direct evidence that p21 protein can be detected in PC3-CAR2 cells 24 h after infecting with AdCMV-p21 at 10 m.o.i. and that the elevated p21 protein levels exhibited in a dose- and time-dependent manner. Similarly, the steady-state level of Rb protein, a key indicator for p21-induced growth inhibition, was reduced, and the majority of Rb protein was hypophosphorylated in PC3-CAR2 cells after infecting with p21 virus (Fig. 1*E*).

Furthermore, the PC3-vector cells with 10% CAR-positive cells (Table 1) exhibited a strong resistance to p21-induced growth inhibition (Fig. 1*B*) because p21 virus failed to infect this clone (Fig. 1*D*). This was evidenced by the presence of hyperphosphorylated Rb protein levels in PC3-vector cells (Fig. 1*E*). Therefore, CAR protein appears a critical rate-limiting factor in determining the outcome of gene therapy.

***In Vitro* Growth Characteristics of CAR-transfected Prostate Cancer Cells.** Structurally, CAR belongs to the immunoglobulin superfamily. It shares similar structure with a cell adhesion molecule such as C-CAM, a potent tumor suppressor in prostate cancer (2, 12). Also, CAR levels in the three human prostate cancer cell lines seem to correlate with their *in vivo* tumorigenicity (15). Therefore, we decided to examine the effect of CAR on the *in vitro* growth rate of PC3 cells. The growth rates of both PC3-CAR2 and -11 cells were ~40% that of PC3-vector cells at day 6 (Fig. 2*B*). The growth rate of both PC3-CAR7 and 14 cells were ~75 and 85% that of PC3-vector

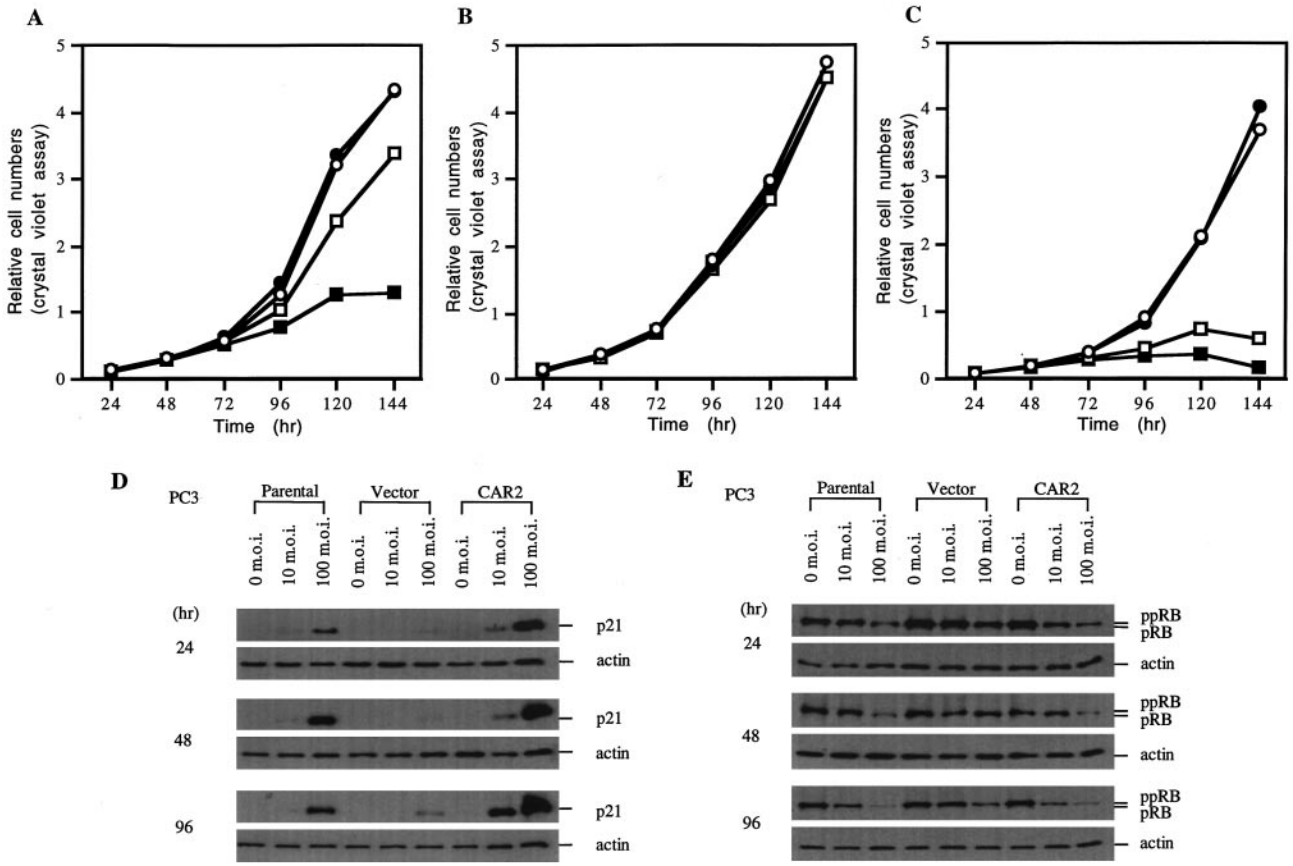

Fig. 1. Increased the efficacy of gene therapy in CAR-expressing prostate cancer. PC3-parental cells, PC3-vector cells, and PC3-CAR2 cells were infected with p21 adenovirus at 0, 1, 10, 100 m.o.i. At the indicated time, total cell number was determined by the crystal violet assay. Protein extracts were analyzed by Western blot analysis with p21- or Rb-specific antibodies. *A*, growth rate of PC3-parental cells infected with p21 virus; *B*, growth rate of PC3-vector cells infected with p21 virus. *C*, growth rate of PC3-CAR2 cells infected with p21 virus; *D*, expression of exogenous p21 proteins in PC3 cells infected by p21 adenovirus; *E*, phosphated Rb levels in p21 virus-infected PC3 clones. *ppRb*, hyperphosphorylated form of Rb; *pRb*, hypophosphorylated form. ○, control; ●, 1 m.o.i.; □, 10 m.o.i.; ■, 100 m.o.i.

cells at day 6, respectively. PC3-CAR2 and 11 cells grow more slowly than PC3-CAR7 and -14 cells because of the higher CAR levels in both PC3-CAR2 and -11. To rule out this result from the artifact of stable transfection, we also examined the growth rate of PC3 cells from transient expression of CAR. As shown in Fig. 3, *A* and *B*, the growth-inhibitory activity of CAR exhibited in a dose-dependent manner. This is not due to the cytotoxicity caused by the presence of the large amount of DNA, because transfecting high concentrations of both cDNA constructs containing oncogenic protein such as EGFR and $p120^{ras}$ resulted in more cells than that of CAR cDNA (Fig. 3*C*). These findings indicate that CAR is a potent tumor inhibitor for prostate cancer.

With respect to the dual function of the CAR molecule, we further examined the structural functional relationship between viral binding and tumor inhibition. The extracellular domain is critical for viral uptake (11). With the same construct (11) containing only the extracellular domain with a glycolipid anchor for membrane attachment, two PC3 sublines (GPI4 and -12) were generated. As shown in Table 1, increased CAR expression was detected, and cells were sensitive to viruses. However, neither subline exhibited growth inhibition under the same experimental condition (Fig. 2*C*). These cells appeared to be tumorigenic *in vivo* (83%). On the other hand, both Tailess CAR cDNA-transfected cells (*i.e.*, Tailess 4 and Tailess 5) appeared to be a potent inhibitor *in vitro* (Fig. 2*C*) and *in vivo* (3%). Therefore, we believe that CAR is a tumor inhibitor; both the extracellular and transmembrane domains of CAR are required.

Suppression of *in Vivo* Tumorigenicity Of PC3 Cells by Increasing CAR Expression. PC3 cells have been shown to be highly tumorigenic when injected into nude mice (12). To test whether increased expression of CAR may affect the tumorigenicity of PC3 cells, cells from each clone, including PC3-parental, PC3-vector, and four clones of PC3-CAR (2, 7, 11, and 14) were injected s.c. into the flanks of male athymic nude mice, and the incidence of tumor formation and the volumes of the tumors were monitored weekly when tumors become palpable. As shown in Table 2, the tumor incidence elicited by PC3-CAR2 showed a significant decrease compared with that by both PC3-parental and PC3-vector. Overall, the decreased tumor incidence elicited by four CAR-transfected PC3 cells (Table 2) correlated with the CAR levels in each clone (Table 1). Table 2 shows the tumor growth results obtained from three independent experiments. Four weeks after injection, four CAR-transfected PC3 clones demonstrated a significant ($P < 0.05$) tumor growth inhibition compared with PC3-vector. Furthermore, 8 weeks after injection, the tumor volume induced by four CAR-transfected PC3 clones was smaller than that induced by both PC3-parental and -vector cells. As determined by cytometric analysis, the tumor volume has an inverse correlation with the CAR levels in each clone (Table 1), indicating that CAR has a dosage effect in suppressing tumor growth. We also noticed that a few tumors induced by PC3-CAR2 are quite large, which suggests that these tumors may be an outgrowth of cells expressing a low level of CAR protein. Using FACS analysis, we measured CAR levels in the cells derived from these tumors (3%,

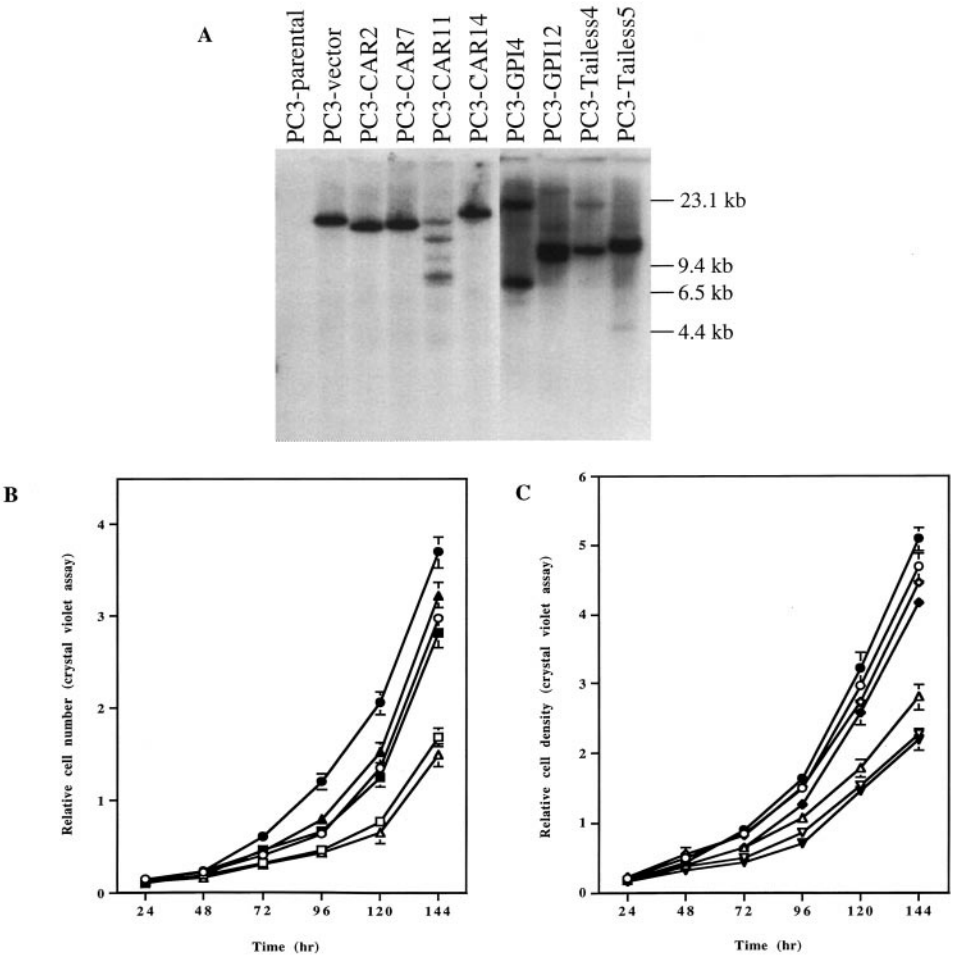

Fig. 2. *In vitro* characterization of CAR-transfected PC3 cells. *A*, high-molecular-weight DNA (20 μg) was digested with *Hin*dIII and subjected to Southern blot analysis probed with a *neo* cDNA probe. In *B* and *C*, cells were plated at a density of 5000 cells/ml in 48-well plates in T medium containing 0.2% FBS. Cells were harvested and counted at the indicated time. The relative cell numbers were determined by crystal violet assay. ○, PC3-parental; ●, PC3-vector; □, PC3-CAR2; ■, PC3-CAR7; △, PC3-CAR11; ▲, PC3-CAR14; ◇, PC3-GPI4; ♦, PC3-GPI12; ▽, PC3-Tailess4; ▼, PC3-Tailess5.

38%, 41%, 54%, 57%) and found a strong inverse correlation between tumor volume (364 mm^3, 260 mm^3, 197 mm^3, 94 mm^3, 33 mm^3) and the CAR levels in each clone. Taken together, we believe that CAR is a potent tumor inhibitor for human prostate cancer.

Discussion

Gene therapy, through either replacing defective or suppressing gene overexpression, is an innovative approach to the treatment of malignant and benign disorders. Cytotoxic genes such as HSV-TK (16) and cytokines for boosting host immunity are good candidates for cancer gene therapy. Several practical and theoretical (17) considerations make recombinant adenovirus an attractive vector for cancer gene therapy. For example, adenoviral infection results in episome DNA replication without the chromosomal integration that may cause potential genotoxicity. A recombinant adenovirus can carry a large of transgene (17, 18); these recombinant adenoviruses are structurally stable, and no genome rearrangement is detected after extensive amplification (19). Also, adenovirus can infect virtually all of the epithelial cells, regardless of their cell cycle stage. Importantly, adenoviral infection appears to be linked only to mild disease, such as acute respiratory disease. However, because viral proteins are cytotoxic and immunogenic, repeated administration of adenovirus may elicit cell-mediated immunity, *i.e.*, infiltration of $CD8^+$ T cell (20). Therefore, an agent that can increase the infectivity of recombinant adenovirus at a lower viral dosage may potentially avoid such adverse side effect while increasing the efficiently of gene delivery.

In recent studies, we reported that a wide spectrum of CAR levels exists among several human bladder cancer lines (8). Using both virus binding and virus infectivity assay, we found that the levels of CAR correlate with viral sensitivity determined. Moreover, we observed loss or reduced expression of CAR levels in several human bladder cancer lines. Using Northern blot and quantitative RT-PCR analyses, we documented that a significant difference in viral receptor levels is caused by down-regulation of the CAR gene in several resistant cancer cell lines. Similarly, in other cancer types such as melanoma and glioma, variable expression of CAR gene is also documented (21, 22). Southern blot analysis indicated that there is no large gene alteration or rearrangement in the CAR gene between the CAR-positive and CAR-negative cells (8). This suggests that transcriptional regulation of the CAR gene is critical for its steady-state levels.

In this study, derived from a patient with a bony metastasis, we demonstrated that CAR protein levels are down-regulated in an androgen-independent human prostate cancer line (PC3). It appears that PC3 cells are resistant to adenoviral infection. To revert this viral resistance, we genetically engineered PC3 cells by increasing 35% of CAR-positive cells to 86% CAR-positive cells, and we were able to detect that transgene activity in CAR-positive cells, such as β-galactosidase, increased about 5-fold compared with CAR-negative cells. We further evaluated the efficacy of gene therapy for PC3-cells using a recombinant AdCMV-p21virus. The elevated levels of p21 protein in virus-infected cells result in the accumulation of hypophosphorylated Rb, G_1 arrest, and apoptosis (23, 24). Data from this study (Table 1 and Fig. 1) show that the CAR-transfected PC3 cells (*i.e.*, PC3-CAR2) exhibited about 10 times the viral sensitivity of PC3-vector cells. With Western analysis (Fig. 1*D*), increased expression of p21 protein in both a dosage- and time-dependent manner were

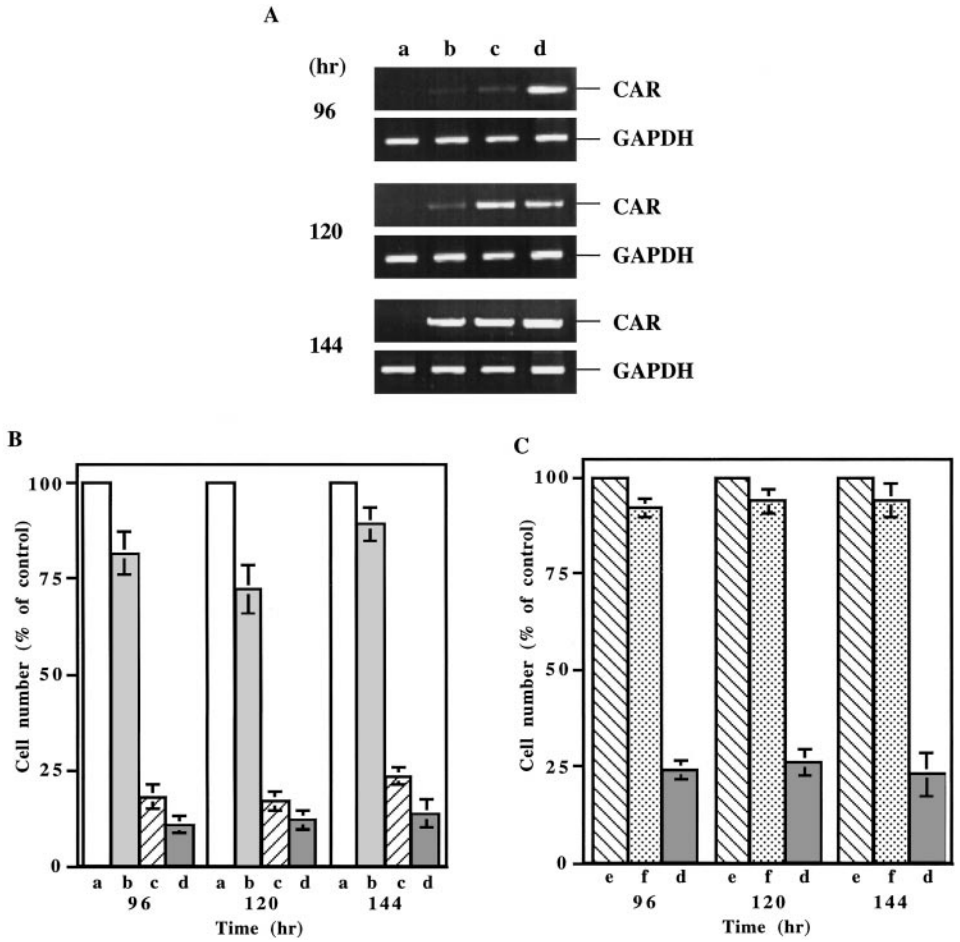

Fig. 3. The growth inhibition of PC3 cells by transient expression of CAR cDNA. The effect of transient expression of CAR on the growth of PC3 cells was determined by the method described by Yeung *et al.* (27) with minor modification. Cells (150,000 cells/p-60 plate) were plated in T-medium containing 5% FBS and cotransfected with different amount of CAR cDNA, β-gal cDNA (0.2 μg), and control plasmid to make the same concentration (1 μg) in each experiment. Twenty-four hours after transfection, cells were changed to T medium containing 0.2% FBS. At the indicated time, the levels of CAR expression were determined by RT-PCR (*A*) and total blue cells were counted (*B, C*). Each data set was repeated in triplicate. The percentage of control was calculated as the number of blue cells from each transfection *versus* either 0 μg of CAR (*B*) or 0.8 μg of EGFR (*C*). CAR: *a*, 0 μg; *b*, 0.1 μg; *c*, 0.4 μg; *d*, 0.8 μg. EGFR: *e*, 0.8 μg. Ras: *f*, 0.8 μg. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

detected in PC3-CAR2 cells, but not in PC3-parental or PC3-vector cells. Concurrently, Rb proteins converted to the hypophosphorylated form were also detected in PC3-CAR2 cells, but not in PC3-parental or PC3-vector cells (Fig. 1*E*). Increased expression of p21 protein in PC3 cells also resulted in apoptosis (data not shown). Taken together, AdCMV-p21 adenovirus-mediated growth inhibition in PC3-cells can be enhanced significantly with an increment of their CAR protein levels. Because a lower dose of adenovirus delivery for CAR-positive cells can achieve the same therapeutic outcome as a higher dose of adenovirus, we suggest that a careful determination of CAR status in target cells must be evaluated before the treatment. By doing so, excessive *in vivo* administration of adenovirus may be avoided.

CAR protein contains two immunoglobulin loops on its extracellular, transmembrane, and intracellular domain (6, 7, 25). Therefore, this protein belongs to the immunoglobulin superfamily. We also noticed that CAR-transfected PC3 cells (72% for PC3-CAR2, 35% for PC3-CAR7) can increase the cell attachment on the plate compared with either PC3-parental (7%) or PC3-vector cells (4%), which indicates that CAR has cell-adhesive activity. Our laboratory has demonstrated that C-CAM1, an immunoglobulin-like cell adhesion molecule, can inhibit tumor growth effectively *in vitro* and *in vivo* (11, 14, 26). Because CAR levels in three human prostate cancer cell line (LNCaP, DU145, PC3) correlate with their *in vivo* tumorigenic potential (11) and CAR is down-regulated in human prostate cancer specimens (data not shown), we were led to examine the biological function of CAR in prostate cancer. Clearly, our results indicate that stable transfection of CAR cDNA could inhibit the *in vitro* growth of PC3 cells (Fig. 2) and that transient expression of CAR can also

Table 2 *Tumor incidence and growth rate of CAR-transfected prostate cancer cells*

			Mean volume ($mm^3 \pm SD$)[b]					
	Tumor incidence (%)[a]		Experiment 1		Experiment 2		Experiment 3	
Clone	28 days	56 days	28 days	56 days	28 days	56 days	28 days	56 days
PC3-parental	44/60 (73)	47/60 (78)	37 ± 8	345 ± 95	32 ± 12	307 ± 81	49 ± 6	265 ± 85
PC3-vector	51/60 (85)	51/60 (85)	46 ± 8	346 ± 63	46 ± 8	374 ± 64	54 ± 13	312 ± 75
PC3-CAR2	24/54 (44)	27/54 (50)	28 ± 5[c]	232 ± 60[c]	18 ± 4[c]	153 ± 25[c]	23 ± 19[c]	168 ± 63[c]
PC3-CAR7	34/54 (63)	34/54 (63)	37 ± 6[d]	302 ± 50[c]	29 ± 12[d]	240 ± 20[c]	41 ± 13[c]	227 ± 72[c]
PC3-CAR11	33/54 (61)	34/54 (63)	29 ± 7[c]	270 ± 59[c]	25 ± 6[c]	214 ± 95[c]	31 ± 11[c]	184 ± 54[c]
PC3-CAR14	35/54 (65)	36/54 (67)	36 ± 10[d]	307 ± 94[c]	22 ± 4[c]	223 ± 22[c]	32 ± 12[c]	211 ± 60[c]

[a] Tumor incidence is calculated from three independent experiments.
[b] Athymic mice were inoculated s.c. with 1×10^6 cells/sites at six sites s.c. in the flanks of the 6- to 8-week-old animals at day 0. Tumor size was determined by the formula length × width × height × 0.5236.
[c] The CAR-transfected PC3 cells showed significant tumor growth inhibition compared with PC3-parental and PC3-vector cells ($P < 0.05$).
[d] The CAR-transfected PC3 cells showed significant tumor growth inhibition compared with PC3-vector cells ($P < 0.05$).

inhibit the cell growth in a dose-dependent manner (Fig. 3). By injecting the CAR-transfected PC3 cell-induced tumors into athymic nude mice s.c., we observed a decrease of *in vivo* tumor incidence and tumor growth rate (Table 2). A similar growth-inhibitory effect of CAR is thus observed in human bladder cancer lines (data not shown). These results demonstrate that CAR expression in prostate cancer cells could be a potent growth inhibitor from *in vitro* and *in vivo*.

However, the mechanism of action of CAR protein in prostate cancer is still unknown. We have recently shown that the intracellular domain but not the immunoglobulin domain of C-CAM1 is crucial for its tumor-inhibitory activity (26). This suggests that the intracellular domain may be able to elicit a signaling pathway in prostate cancer. Interestingly, our data indicate that the extracellular domain of CAR is essential for viral infection (Table 1). In addition to the extracellular domain of CAR, the transmembrane domain is required for growth inhibition (Fig. 2*C*). It is possible that the transmembrane domain of CAR can interact with other peripheral proteins associated with membrane that leads to signal transduction. The analysis of detailed mechanism is under way. Nevertheless, all of the information derived from further study can be translated into the development of CAR as a new agent for improving gene therapy.

In conclusion, our findings indicate that increased expression of CAR protein can inhibit tumors growth *in vitro* and *in vivo*. The tumor-suppressing effect of CAR and its adenoviral receptor nature indicate that CAR has a dual effect to potentiate prostate cancer gene therapy. We therefore believe that CAR proteins have significant biological and therapeutic implications for human prostate cancer. Further study for up-regulating the endogenous CAR gene in prostate cancer will make a significant contribution not only to the prostate cancer therapy but also to the application of gene therapy of other cancer types.

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