Effects of an adenoviral vector containing a suicide gene fusion on growth characteristics of breast cancer cells

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Abstract. The herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) and the cytosine deaminase/5-fluorocytosine (CD/5-FC) systems have been widely applied in suicide gene therapy for cancer. Although suicide gene therapy has been successfully used *in vitro* and *in vivo* studies, the number of studies on the effects of recombinant adenoviruses (Ads) containing suicide genes on target cancer cells is limited. The aim of this study was to examine whether recombinant Ads containing the *CD/TK* fusion gene affect cell proliferation of breast cancer cells *in vitro*. In the present study, we explored the use of a recombinant adenoviral vector to deliver the *CD/TK* fusion gene to the breast cancer cell line MCF-7. We found that the recombinant adenoviral vector efficiently infected MCF-7 cells. Western blot analysis revealed that CD and TK proteins are expressed in the infected cells. The infected breast cancer cells did not show any significant changes in morphology, ultrastructure, cell growth, and cell-cycle distribution compared to the uninfected cells. This study revealed that the Ad-vascular endothelial growth factor promoter (VEGFp)-CD/TK vector is non-toxic to MCF-7 cells at the appropriate titer. Our results indicate that it is feasible to use a recombinant adenoviral vector containing the *CD/TK* fusion gene in suicide gene therapy to target breast cancer cells.

Introduction

The cytosine deaminase/5-fluorocytosine (CD/5-FC) and the thymidine kinase/ganciclovir (TK/GCV) are the most common suicide gene therapy systems (1,2). Several studies have adopted strategies making use of the CD/5-FC and the herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) systems, which are more effective when combined as compared to their use alone (3-5). However, relatively few reports exist on the effect of the vector combining the two systems on cancer cells. Therefore, we initiated experiments to assess the effect of a recombinant adenovirus (Ad) containing the *CD/TK* fusion gene on MCF-7 cells, in order to contribute to the development of new strategies for the improvement of the clinical application of current double suicide gene therapy protocols.

Recombinant adenoviral (Ad) vectors have been widely used as a gene delivery vehicle, since they can efficiently transfer genes into a wide spectrum of cell types at a high efficiency *in vitro* and *in vivo* (6-8). One of the major goals of cancer gene therapy is to increase selective death of cancer cells. To achieve this, a number of methods have been adopted, based on either cell type-specific receptors allowing targeted gene delivery, or tissue-specific promoters allowing heterologous gene expression in specific organs (9-11); these methods have in general proven satisfactory *in vitro*. Based on the overexpression of the vascular endothelial growth factor (VEGF) in breast cancer cells and the absence of its expression in healthy breast tissues (12,13), a recombinant Ad carrying the *VEGF* gene promoter and the *CD/TK* fusion gene, named Ad-VEGFp-CD/TK, was previously constructed by our group (14). This vector is expected to allow specific expression of the fusion suicide gene (*CD/TK*) via the *VEGF* promoter.

In the present study, we investigated whether the suicide gene fusion *CD/TK* driven by the *VEGF* promoter can achieve high-efficiency gene transfer and high-level expression of the CD and TK proteins in MCF-7 breast cancer cells. Moreover, we studied the effects of the recombinant Ad containing the *CD/TK* gene fusion on the morphology and growth characteristics of breast cancer cells. We investigated the feasibility of the approach using the *VEGF*-driven *CD* and *TK* genes to target breast cancer cells *in vitro*. Since *VEGF* is overexpressed in numerous solid tumors, this approach may improve selectivity towards cancer cells and may be applicable on a wide range of tumors.

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Materials and methods

Cell culture. The human embryonic kidney epithelial 293 (HEK-293) and the breast cancer MCF-7 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). As previously described (14), MCF-7 cells and HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen Life Technologies) in an incubator at 5% CO_2, at 37˚C. When the cells had reached 90% confluence, they were digested into single cell suspensions using trypsin (Amresco, Solon, OH, USA). Cells were harvested and subcultured. Subsequently, cells in logarithmic growth phase were selected for investigation.

Recombinant adenoviral vector. The recombinant Ad carrying the *VEGF* gene promoter and the *CD/TK* fusion gene, named Ad-VEGFp-CD/TK, was previously constructed and preserved by our group (14). The vector also contains the green fluorescence protein reporter gene (*GFP*), which was used as a marker for the delivery of target genes into MCF-7 cells. The vector was repeatedly transfected into HEK-293 cells to allow amplification. Next, the Ads were purified by caesium chloride gradient ultracentrifugation at 32,000 x g, at 15˚C, for 1 h. Subsequently, the adenoviral titers were determined with the endpoint dilution assay and the plaque forming units (pfu) were assessed with a plaque assay, as described in (14,15), respectively. The titer of Ad-VEGFp-CD/TK was $2.2x10^{11}$ pfu/ml.

Adenoviral infection. Four million MCF-7 cells were inoculated in each well of 6-well plates. Cultures were maintained for 12 h, and cells were then infected with Ad-VEGFp-CD/TK at multiplicities of infection (MOI) of 20, 40, 60, 80, 100 and 200 pfu/cell, for 24 h. The number of GFP-positive cells was counted under an inverted fluorescence microscope (Leica, Mannheim, Germany).

Microscopy and cellular morphology. The experimental group was infected for 24 h with the adenoviral vector at MOI 100. The control group was cultured in DMEM for 24 h. One day later, the cells in the two groups entered the logarithmic growth phase. The morphological changes of MCF-7 cells were examined under a phase contrast light microscope (Leica, Mannheim, Germany).

Transmission electron microscopy (TEM). MCF-7 cells were incubated overnight in a 75-ml cell culture bottle. Then, the experimental group was incubated with the adenoviral vector Ad-VEGFp-CD/TK for 72 h at 37˚C with 5% CO_2. The control group was cultured in DMEM for 72 h. The cultured cells were harvested using trypsin and centrifuged for 10 min at 2,000 x g at room temperature. The pellets were next fixed overnight in 3% (v/v) glutaraldeyde at 4˚C. The specimens were washed in phosphate-buffered saline (PBS) and post-fixed in 1% osmium tetraoxide for 20 min. Then, the specimens were dehydrated in a graded series of acetone dilutions. The area of interest in the resin block containing the embedded cells was selected using toluidine blue staining (Polysciences, Warrington, PA, USA), and later examined under a light microscope. Ultrathin sections of the selected area were performed using a Leica EM UC7 ultramicrotome (Leica). The stained samples were then observed using TEM (Philips, Eindhoven, The Netherlands). The nucleus-to-cytoplasm ratio was evaluated using the ratio of the volume size of the cell nucleus to the volume size of the cell cytoplasm.

Western blot analysis. MCF-7 cells were inoculated on 90-mm dishes and transduced with recombinant Ads for 24 h using the protocol of adenovirus vector infection. Cells were then lysed using lysis buffer (50 mM Tris/pH 8.0, 150 mM NaCl, 1% (w/v) Triton-X-100, 0.1% (w/v) SDS, 1% (w/v) sodium deoxycholate) and proteins were extracted from the MCF-7 cell lysate by centrifugation (Sigma, Deisenhofen, Germany) at 14,000 x g for 10 min. The extracted proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred onto a polyvinylidene fluoride membrane. Subsequently, the membrane was incubated with 30 g/l non-fat milk, and next, with sheep anti-CD (Biogenesis, Poole, UK) or goat anti-TK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibodies overnight at 4˚C. After a wash in Tris-buffered saline with Tween-20 (TBST), horseradish peroxidase-labeled rabbit anti-sheep or anti-goat IgG was added as the secondary antibody (Santa Cruz Biotechnology, Inc.), and incubated at room temperature for 1 h. The membrane was washed with TBST, and incubated with an enhanced chemiluminescence substrate (ECL; Merck KGaA, Darmstadt, Germany) for 1 min. Finally, the membrane was developed on an X-ray film (Fujifilm, Tokyo, Japan).

Cell growth curve. Transfected MCF-7 cells were inoculated on 24-pore plates at a density of $1x10^4$/pore. The control group was untransfected MCF-7 cells. Three parallel pores were assayed for each group. MCF-7 cells were observed and counted each day for 7 consecutive days in order to establish the growth curve.

Flow cytometry (FCM) analysis. FCM analysis was used to assess the distribution of MCF-7 cells at the different cell cycle stages, as in (16). Briefly, $5x10^5$ MCF-7 cells at the logarithmic growth phase were inoculated in a 50-ml cell culture bottle. The experimental group was cultured with the adenoviral vector (Ad-VEGFp-CD/TK) for 72 h at 37˚C with 5% CO_2. The control group was cultured in DMEM for 72 h. The cultured cells were harvested using trypsin and washed in PBS. The pelleted cells were later fixed in cold absolute ethanol (final concentration, 70%) overnight at 4˚C. The cell suspension was rewashed using PBS and centrifuged at 1,000 x g for 10 min. After the ethanol was discarded, 200 μl of RNase (1 mg/ml; Sigma, St. Louis, MO, USA) were added to the pellet, which was gently mixed. The samples were kept at 37˚C for 60 min. Subsequently, 800 μl of propidium iodide (0.5 mg/ml; Sigma) were added to 400 μl of each cell suspension, and were left to incubate for 30 min at 4˚C. FCM was then performed on a FACSCaliber cytometer (Becton Dickinson, San Jose, CA, USA). The ModFit LT software (Verity, Topsham, ME, USA) was used for data quantification.

Statistical analysis. The experimental data were processed with the SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Data were expressed as mean ± standard error of the mean (SEM).

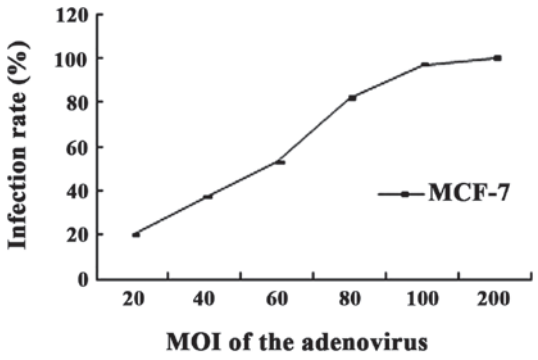

Figure 1. Percentage of infected cells based on multiplicities of infection (MOI).

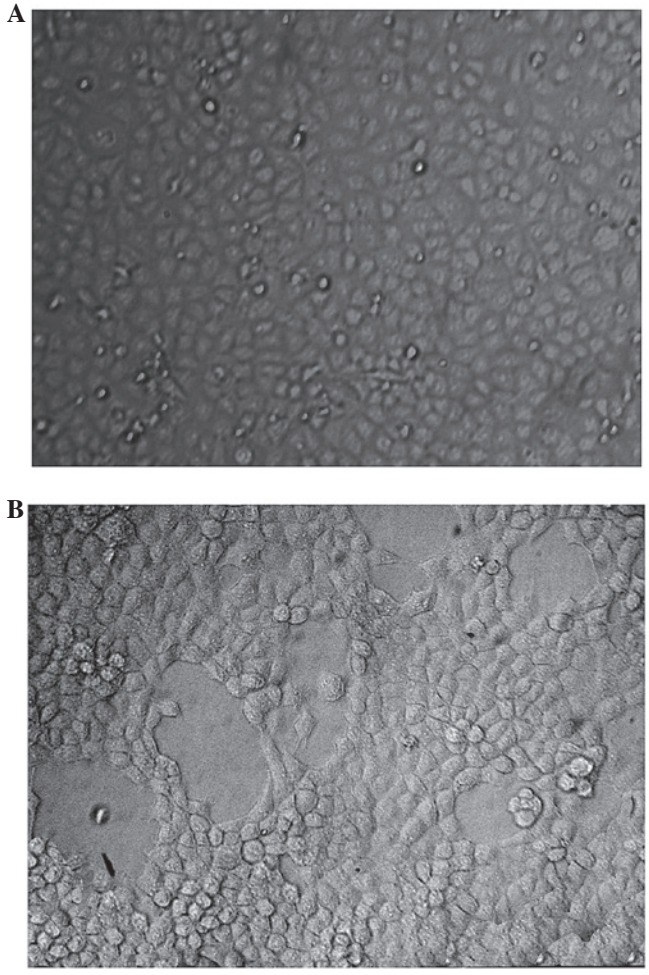

Figure 2. Morphology of MCF-7 cells prior to and following infection with the adenovirus, as observed under a phase contrast microscope (magnification, x200). (A) Non-transfected cells and (B) experimental group of cells transfected with the adenoviral vector for 24 h.

An independent samples t-test was used to compare the means between two groups. $P<0.05$ was considered to indicate statistically significant differences.

Results

Effective infection of the adenoviral vector in MCF-7 cells. We estimated the transduction efficiencies of the adenoviral-mediated gene transfer in human breast cancer cells infected at different MOI of the Ad vector. As the MOI increased, the percentage of infected cells also increased (Fig. 1). At MOI of 100 and 200 pfu/cell, >95% of MCF-7 cells were GFP-positive, without any obvious toxicity effects observed. Therefore, we concluded that the adenoviral vector efficiently infects the human breast cancer cells *in vitro*.

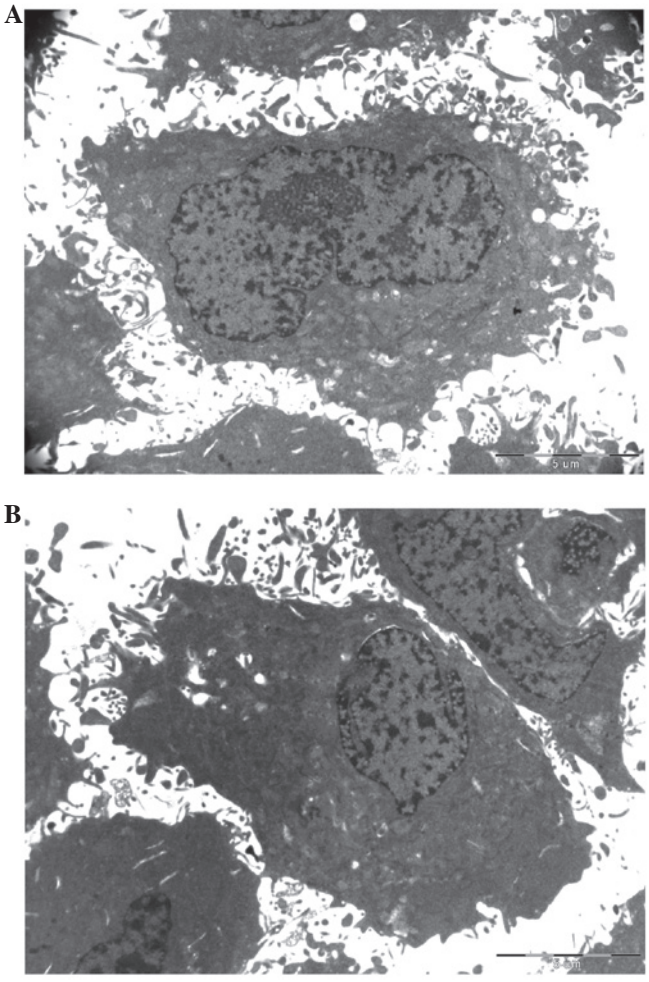

Figure 3. Ultrastructural features of MCF-7 cells prior to and following infection with the adenovirus, as observed under an electron microscope (magnification, x8,000). (A) Control group, untransfected cells and (B) experimental group, cells transfected with the adenovirus for 72 h.

Effect of infection on cell morphology. MCF-7 cells were treated with the adenoviral vector at MOI 100 for 24 h. The infection toxicity was then evaluated by observations of the cell morphology (Fig. 2). No change in cellular morphology was observed after the 24-h treatment of the cells with the adenoviral vector (Fig. 2B). The adherent cells typically show a polygonal, spreading shape, with a large nucleus. However, we found that cell densities in the experimental group (transfected cells) were reduced compared to the control group. We explored whether cell density may be associated with the cell proliferation rate by cell growth and cell cycle analyses described below.

Ultrastructure of MCF-7 cells. As revealed by TEM, the ultrastructure of the transfected MCF-7 cells (Fig. 3B,

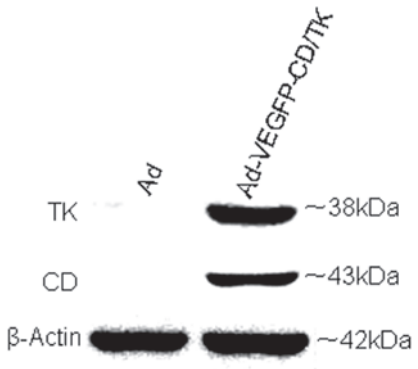

Figure 4. Western blot analysis shows that cytosine deaminase (CD) and thymidine kinase (TK) are expressed in protein extracts prepared from MCF-7 cell lysates of infected MCF-7 cells, but not of control cells.

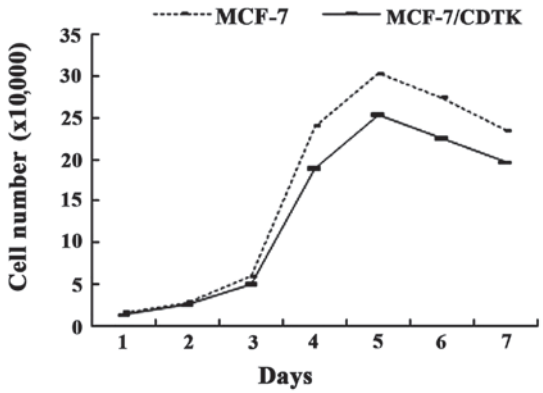

Figure 5. Growth curves of MCF-7 cells and of MCF-7 cells transfected with the adenoviral vector (MCF-7/CDTK).

experimental group) was similar to that of the non-transfected MCF-7 cells (Fig. 3A, control group). The centrally placed nucleus commonly contained one or two prominent nucleoli. The nucleus-to-cytoplasm ratio was 1:1.

Expression of CD and TK proteins. The expression of the CD and TK proteins was analyzed by western blot analysis. The results showed that the CD and TK proteins are not expressed in the control group, while they were strongly expressed in the experimental group (Fig. 4).

Cell growth curve of MCF-7 cells. MCF-7 cells were observed under a light microscope following an additional 72-h incubation with the adenoviral vector. The morphology of MCF-7 cells of the experimental group was similar to that of the control group. The growth of cells became relatively slow at one to two days after infection, while growth rates increased after two days. An increasing number of cells became cell growth arrested and eventually senescent from the fifth day onwards. Cell proliferation of the experimental group was reduced compared to the control group (Fig. 5). The data from cell growth experiments were statistically analyzed using an independent samples t-test. There was no significant difference in cell proliferation between the two groups ($P>0.056$). This result showed that the adenoviral vector has no obvious effect on MCF-7 cell growth (Table I and Fig. 5).

Cell cycle analysis using FCM. The proportion of cells at the different phases of the cell cycle is shown in Table II. The data from the experimental and the control group were statistically analyzed by an independent samples t-test, which revealed no significant differences ($P>0.085$) in the number of cells at the same phase of the cell cycle between the two groups (Table II). In addition, FCM analysis revealed that the adenoviral vector has no obvious effect on the cell cycle distribution of MCF-7 cells (Fig. 6).

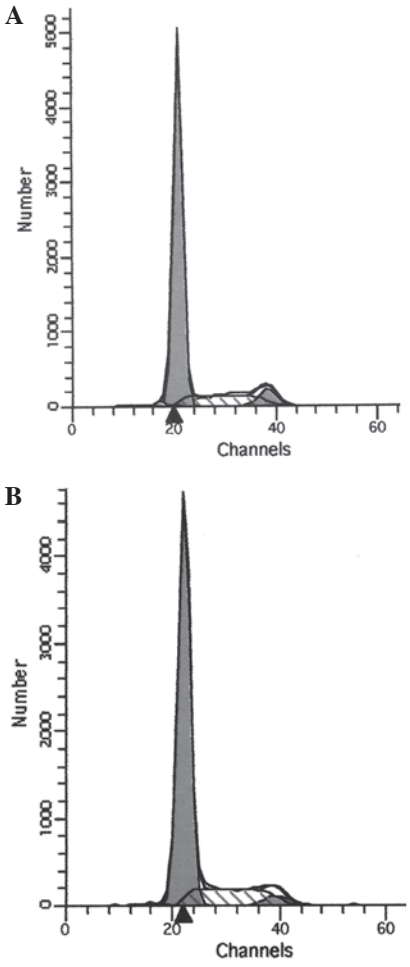

Figure 6. Changes in the cell cycle of (A) untransfected and (B) transfected MCF-7 cells, as examined by flow cytometry.

Discussion

Adenoviral vectors are popular gene delivery vectors in clinical trials for gene therapy (17-19). There are several advantages of using adenoviral vectors: first, the efficiency of transduction is high, as is the level of gene expression (20,21). In this study, we used an adenoviral vector containing the *GFP* reporter gene, which is a commonly used reporter allowing to unequivocally assess whether a gene is expressed. GFP expression was evaluated by fluorescence microscopy, which allowed to assess the efficiency of transduction. This experiment demonstrated that >95% of the cells were GFP-positive at a MOI of 100 and 200 pfu/cell. The recombinant adenoviral vector showed no

Table I. Number of MCF-7 cells at different days of infection (mean x10^5 ± SD, n=3).

	Days of infection						
Group	1	2	3	4	5	6	7
MCF-7	1.56±0.14	2.80±0.26	5.97±0.58	23.99±2.92	30.32±2.95	27.44±2.58	23.45±2.09
MCF-7/CDTK	1.31±0.11	2.56±0.19	5.01±0.27	18.90±2.35	25.35±2.14	22.51±1.92	19.64±1.67
t	2.463	1.265	2.574	2.349	2.363	2.659	2.469
P	0.069	0.274	0.062	0.079	0.077	0.056	0.069

SD, standard deviation; MCF-7/CDTK, MCF-7 cells infected with the adenoviral vector; t, a parameter based on an analysis of t-test; P>0.05, between the MCF-7/CDTK group and the MCF-7 group.

Table II. Cell cycle changes in MCF-7 cells following infection with the adenoviral vector (mean% of cells ± SD, n=3).

Group	G_0-G_1	G_2-M	S
Control	77.03±3.27	7.89±1.43	15.01±1.41
Transfected	73.55±7.34	10.77±1.66	15.46±1.53
t	0.749	2.279	0.375
P	0.496	0.085	0.727

SD, standard deviation; control; untransfected cells; t, a parameter based on an analysis of t-test; P>0.05 between the transfected group and the control group.

toxicity to MCF-7 cells at M0I 100 and 200 (Fig. 2B and C). Second, adenoviral vectors can accommodate relatively large segments of DNA (up to 7.5 kb), which they can transduce into the target cells. In this study, the *CD*/*TK* fusion gene was 2.4 kb. The CD and TK proteins were successfully expressed in MCF-7 cells infected with the adenoviral vector, as shown by western blot analysis (Fig. 4). A major disadvantage of current adenoviral vectors is their cytotoxicity to target cells. Teramoto *et al* (22) showed that adenoviral vectors directly alter the cell cycle in the infected airway cells, which may result in slower proliferation: slower proliferation and cell apoptosis were observed in cells infected by vector at a MOI of 10^4. Slower proliferation and cell apoptosis following Ad vector administration may be due to the high adenoviral titer. If we select an appropriate adenoviral titer, cell proliferation may be not affected, as discussed below.

The VEGF protein has been shown to be upregulated in numerous types of cancer (23,24), including human breast cancer cells (25,26). A VEGF promoter-based adenoviral vector strategy has been successfully adopted to introduce a foreign gene into cancer cells (27,28). However, this strategy has not been systematically explored in breast cancer research. Our study will thus provide valuable information on the use of adenoviral vectors for breast cancer therapy. Our previous studies successfully constructed and extensively analyzed the adenoviral vector Ad-VEGFp-CD/TK (14,29). In the present study, we compared the expression levels of CD and TK protein (using β-actin as the loading control) between untransfected and transfected MCF-7 cells by western blot analysis. This analysis showed that the expression levels of CD and TK in transfected cells are similar to the expression level of β-actin. Thus, the VEGF promoter activated CD and TK protein expression in MCF-7 cells. We conclude that VEGF promoter-based Ads have good potential to be developed as effective therapeutic agents for cancer.

This study indicated that infection with the Ad-VEGFp-CD/TK vector exerts no prominent effect on cell proliferation in the human breast cancer cell line MCF-7 at a MOI of 100. Therefore, MOI 100 was selected as the working concentration. We found that infected MCF-7 cells have a lower growth rate than the uninfected cells. However, there was no significant difference (P>0.05) in cell proliferation between these two groups (Table I). The result from cell growth analysis was consistent with that of cell cycle analysis with flow cytometry: no significant difference (P>0.05) was observed between the two groups with regards to the number of cells at the same phase of the cell cycle (Table II). It is thus reasonable to hypothesize that the adenoviral vector does not alter the cell cycle in the infected MCF-7 cells, which results in the unchanged proliferation of these cells. In addition, no significant change in cell morphology or ultrastructure was observed under the light and the transmission electron microscope. These results indicate that the vector Ad-VEGFp-CD/TK is non-toxic to MCF-7 cells. Three additional studies have successfully used this vector (30-32). Therefore, our study provided the missing information needed for further use of the vector in the context of suicide gene therapy for cancer.

In summary, we achieved high-efficiency transduction of MCF-7 cells by a VEGF promoter-based adenoviral vector, and stable expression of the CD and TK proteins *in vitro*. There were no significant changes in cell morphology, ultrastructure, proliferation rate and cell-cycle distribution in the infected MCF-7 cells. We conclude that the VEGF promoter-based suicide gene system may be an effective strategy for cancer treatment, and that the Ad-VEGFp-CD/TK vector is non-toxic to MCF-7 cells at the appropriate titer.

Acknowledgements

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