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The Use of the L-Plastin Promoter for Adenoviral-mediated, Tumor-specific Gene Expression in Ovarian and Bladder Cancer Cell Lines[1]

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ABSTRACT

A 2.4-kb truncated L-plastin promoter was inserted either 5′ to the *LacZ* gene (Ad-Lp-LacZ) or 5′ to the *cytosine deaminase* (*CD*) gene (Ad-Lp-CD) in a replication-incompetent adenoviral vector backbone. Infectivity and cytotoxicity experiments with the LacZ and CD vectors suggested that the L-plastin promoter-driven transcriptional units were expressed at much higher levels in explants of ovarian cancer cells from patients and in established ovarian or bladder cancer cell lines than they were in normal peritoneal mesothelial cells from surgical specimens, in organ cultures of normal ovarian cells, or in the established CCD minimal deviation fibroblast cell line. Control experiments showed that this difference was not attributable to the lack of infectivity of the normal peritoneal cells, the normal ovarian cells, or the minimal deviation CCD fibroblast cell line, because these cells showed expression of the *LacZ* reporter gene when exposed to the replication-incompetent adenoviral vector carrying the cytomegalovirus (CMV)-driven *LacZ* gene (Ad-CMV-LacZ). The Ovcar-5 and Skov-3 ovarian cancer cell lines exposed to the Ad-Lp-CD adenoviral vector were much more sensitive to the prodrug 5-fluorocytosine (5FC), which is converted from the 5FC prodrug into the toxic chemical 5-fluorouracil, than was the CCD minimal deviation fibroblast cell line after exposure to the same vector. A mouse xenograft model was used to show that the Ad-Lp-CD vector/5FC system could prevent engraftment of ovarian cancer cells in nude mice. Finally, injection of the Ad-Lp-CD vector into s.c. tumor nodules generated a greater reduction of the size of the tumor nodules than did injection of the Ad-CMV-LacZ vectors into tumor nodules. The Ad-Lp-CD vectors were as suppressive to tumor growth as the Ad-CMV-CD vectors. These results suggest that an adenoviral vector carrying the *CD* gene controlled by the L-plastin promoter (Ad-Lp-CD) may be of potential value for the i.p. therapy of ovarian cancer.

INTRODUCTION

Adenoviral vectors are currently among the most frequently used vectors in the gene therapy of cancer because of their high titers, ease of production, high infection efficiency for epithelial neoplastic cells, and the fact that their transcriptional units can be expressed extrachromosomally in nondividing cells. A possible disadvantage of this vector is that its broad host range also results in infection of both the intended tumor cells as well as of the surrounding normal tissues (1–3). This limits the utility of these vectors, especially when the vector gene products are designed to sensitize tumor cells to chemotherapy or to radiation therapy, because of the unwanted toxicity thereby generated in the normal cells.

One way to circumvent this limitation would be to use a tissue-specific transcriptional promoter active only in the target tumor cells. Our laboratory has constructed adenoviral vectors in which the L-plastin promoter is used to activate the expression of therapeutic transgenes in neoplastic but not in normal epithelial cells. L-plastin, which belongs to a family of genes which encode actin-binding proteins, was discovered by Leavitt (4) and his colleagues (5, 6). The only normal cell in which this protein is detectable is the mature leukocyte. This protein has been demonstrated to be present in >90% of epithelial neoplastic cells and is not found in normal epithelial cells. Therefore, the L-plastin promoter may be of potential utility in cancer gene therapy because it can be used to drive the expression of heterologous genes in a tumor-specific manner in the context of recombinant adenoviral vectors. Chung *et al.*, in our laboratory, had reported previously that the *LacZ* gene, when driven by the L-plastin promoter, is expressed in ovarian cancer cells, but not in normal mesothelial peritoneal cells, obtained at the time of surgical resection of ovarian cancer from patients (7).

We now are reporting the results of experiments based on replication-deficient adenoviral vectors that contain either a *LacZ* reporter gene or a *CD*[3] therapeutic transcriptional unit regulated by a 2.4-kb fragment of the L-plastin promoter in bladder and ovarian cancer cell lines, in explants of normal and neoplastic ovarian primary tissue in organ culture, and in ovarian cancer established cell lines in a nude mouse-human tumor xenograft animal model. *CD* is a bacterial gene which converts 5FC, which is nontoxic to cell lines and primary cells, into 5FU, a compound which is toxic to most cells (2, 8). The levels of phosphorylated 5FU generated within *CD*-positive cells are sufficiently high that even nondividing cells die because of disruption of mRNA processing and protein synthesis.

The results of these experiments have shown that:

(*a*) the level of the L-plastin promoter driven the *LacZ* heterologous reporter gene expression is lower in an established minimal deviation fibroblast cell line (CCD) when compared with a collection of established epithelial tumor cell lines derived from ovarian cancer and bladder cancer;

(*b*) the L-plastin promoter activates the *LacZ* and CD transcriptional units to a higher level in ovarian cancer cells than in monolayer and organ explant cultures of normal ovarian tissue or of normal peritoneal tissue; and

(*c*) the cytotoxic effect of replication-incompetent adenoviral vectors carrying the CD transcriptional unit driven by the L-plastin promoter is greater to ovarian cancer cells exposed *in vitro* to 5FC than to explants of normal peritoneum. In addition, the suppressive effect of the L-plastin-driven CD vectors on the *in vivo* growth of ovarian cancer cell lines is equal to that of the CMV-driven CD

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[3] The abbreviations used are: CD, cytosine deaminase; 5FC, 5-fluorocytosine; 5FU, 5-fluorouracil; CMV, cytomegalovirus; NBCS, new born calf serum; CAR, coxsackie B/adenovirus receptor; pfu, plaque-forming units; MOI, multiplicity of infection; X-Gal, (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ONPG, *O*-nitropheny-β-D-galactopyranoside.

vectors. These results suggest that adenoviral vectors carrying the CD transcription unit driven by the L-plastin promoter may be of use in the i.p. treatment of metastatic ovarian cancer.

MATERIALS AND METHODS

Cells and Cell Culture

Human bladder carcinoma cell lines (J82 and EJ) were obtained from Dr. Richard Cote of the University of Southern California, Los Angeles, CA. The CCD minimal deviation human fibroblast cell line, the 293 transformed human kidney cell line, and the Skov-3 human ovarian cancer cell line were obtained from American Type Culture Collection. The Hey cystadenocarcinoma papillary ovarian cancer cell line was obtained from Eva Sapi of the Department of Therapeutic Radiology at Yale University (New Haven, CT). J82, EJ, Hey, and 293 cells were propagated in DMEM (Life Technologies, Inc.) supplemented with 10% heat-inactivated NBCS obtained from Hyclone Laboratories, Inc. (Logan. UT). The Ovcar-5 human epithelial ovary carcinoma cell line was obtained from Dr. Thomas C. Hamilton of the Fox Chase Cancer Center, Philadelphia, PA. Ovcar-5 cells were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% heat-inactivated NBCS. The Skov-3 human ovarian adenocarcinoma cell line was propagated in McCoy5A medium supplemented with 10% heat-inactivated NBCS. All cell cultures were maintained in a 5% CO_2, humidified tissue culture incubator at 37°C.

Chemicals and Reagents

5-FC, 5FU, fluorescein di-β-D-galactopyranose, and X-Gal were purchased from Sigma Chemical Co. The β-Galactosidase Assay Kit was purchased from Stratagene Company. 6-^{3}(H)5-fluorocytosine (4.1 Ci/mmol) and 6-^{3}(H)5-fluorouracil were purchased from Noravek Biochemicals Inc. of Brea, CA. Monoclonal antibodies to αvβ3 (LM609) and αvβ5 (P1F6) integrins were purchased from Chemi-Con International. A monoclonal antibody to the CAR, which binds the adenoviral fibrillar protein, was obtained from Dr. R. W. Finberg of the Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA.

Construction of Replication-incompetent Recombinant Adenoviral Vectors

The Ad-CMV-CD vector, which contained the *CD* gene controlled by a CMV promoter (7) in place of the adenoviral *E1A* and *E1b* genes, was obtained from the laboratory of Dr. Ron Crystal of the Cornell Medical School, New York, NY (8, 9). A similar adenoviral vector (Ad-CMV-LacZ) was engineered in our laboratory in which a β-galactosidase transcriptional unit was inserted into the E1a and E1b regions of the adenoviral vector backbone (7). Injae Chung of our laboratory truncated the 5-kb L-plastin promoter to a 2.4-kb fragment, which extended from nucleotide −2265 of the 5′ region of the L-plastin promoter to +18 bp from the transcription initiation site of the *L-plastin* gene (7). The number of infectious adenoviral particles, expressed as plaque-forming units (pfu) present in the viral stocks, was determined by limiting dilution assay of plaque formation in 293 cells exposed to various dilutions of the vector (10, 11).

Analysis of Cellular Receptors on Tumor Cells That Participate in Vector Uptake

Mouse monoclonal antibodies to the αvβ3 (LM609) integrin and the αvβ5 (P1F6) integrin and to the CAR receptors were used to detect the density of the human αvβ3, αvβ5, and CAR receptors on the test cells. The FACS Star Flow Cytometer (Becton Dickinson) in the Yale Cancer Center FACS Core Laboratory (New Haven, CT) was used to determine the percentage of cells positive for each receptor.

β-Galactosidase Activity Assay

X-Gal Staining. Cells were washed in PBS, trypsinized, and the viable cell number determined by trypan blue exclusion using a light microscope. Cells (3×10^5) for each cell line were infected with varying ratios of pfu/cell (MOI) of the vector in DMEM supplemented with 2% NBCS for 90 min. After this, the cells were plated in six-well plates in complete medium in duplicate cultures. After 48 h of incubation at 37°C in a 5% CO_2, humidified tissue culture incubator, the cells were fixed with ice-cold 2% paraformaldehyde/0.2% glutaraldehyde for 10 min. The level of β-Gal-expression cells was then assessed by staining the cultures with X-Gal and potassium-ferricyanide/ferrocyanide solution essentially as described previously (12, 13). The average number of β-Gal-expressing (blue) cells/well was determined by counting five separate microscopic high-power fields.

β-Galactosidase Assay (ONPG). Cells (5×10^5) were infected at 20 MOI with Ad-Lp-LacZ or Ad-CMV-LacZ in 2% serum for 90 min. PBS was used to wash the cells, which were seeded in six-well plates with the fresh culture medium. The cells were then incubated for 48 h, after which the β-galactosidase assay was conducted (β-Galactosidase Assay Kit, Stratagene). Briefly, the cells were washed in PBS and lysed in 200 μl lysis buffer and the cell debris removed by centrifugation for 5 min. The cell lysate was diluted 10 times, and 15 μl of the cell lysate were pipetted into a 96-well microtiter dish, 145 μl of buffer A-β-mercaptoethanol mixture was added to each well with subsequent incubation for 5 min at 37°C. Fifty μl of ONPG were added to each well, and the dish was incubated at 37°C for 25 min; the mixtures turned bright yellow. The reaction was terminated by adding 90 μl of stop solution and the microtiter dish was scanned in the microtiter dish reader set at 405 nm, and the absorbance (OD) was determined.

The Effect of 5-FU Released from CD Vector-infected Cells on Uninfected Cells

To quantify the effect of 5-FU released from infected cells on uninfected cells, different cell lines were infected at varying MOI (20 MOI, 80 MOI, and 160 MOI) using the Ad-CMV-CD or Ad-Lp-CD vectors. The infected cells and the noninfected cells were mixed in varying ratios to generate 0, 5, 10, 20, 30, 40, 50, 60, and 100% infected cells (14, 15). Cells were then seeded in duplicate in six-well tissue culture plates and incubated for 24 h with subsequent incubation with 500 μM/liter 5FC for 5 days. The number of surviving cells was determined using trypan blue exclusion.

Comparison of the 5-FU Sensitivity (IC_{50}) of Ovarian Cancer and Bladder Cancer Cell Lines with CCD (Minimal Deviation Fibroblast Cell Lines)

The concentrations of 5FU used for the cytotoxicity test (IC_{50}) were 100, 50, 10, 1, and 0.5 μM. After 96 h, the cells were removed with trypsin-EDTA and the cell number calculated using the Coulter Counter ZM (Hialeah, FL).

The Toxicity of Adenoviral Vectors

Cells (2×10^5) were infected with the Ad-CMV-LacZ, Ad-Lp-LacZ, Ad-CMV-CD, or Ad-Lp-CD vectors at MOI of 0, 5, 20, 40, 80, and 160 for 90 min and then seeded in six-well plates in duplicate. Twenty-four h later, 0.5 mM 5FC was added to each well, and then the cells were incubated for 5 days. Then the cells were trypsinized, and the surviving cells were counted using trypan blue exclusion (16). We arbitrarily assigned a 100% value to the cells incubated at 0 MOI and calculated the percentage of viable cells in the cultures to which vector had been added.

Vector Studies in Monolayer Explant Culture

Biopsy samples were cut into small pieces. These pieces were then digested with collagenase to disaggregate the tissue. To test the sensitivity of the patient samples to infection and 5FC sensitization with the Ad-CMV-CD and Ad-Lp-CD vectors, the cells were grown in T25 flasks to 90% confluence. Then the cells were washed in PBS and exposed to vector directly for 90 min in the flasks containing DMEM supplemented with 2% NBCS. Then the cells were incubated for 5 days at 500 μM/liter 5FC concentration, and the cell viability was determined by light microscopic examination.

Vector Studies on Organ Culture of Ovarian Cancer and Normal Ovarian Tissue

Each specimen was cut into pieces of approximately 1–2 mm^3 and immersed in 4 ml of DMEM:Ham's F12 medium, which was supplemented with 10% charcoal-stripped serum (17). Cultures were incubated at 37°C in six-well

plates on a shaking platform for 24–48 h, after which the tissues were exposed to the Ad-CMV-LacZ or Ad-LP-LacZ viral vectors for 90 min in serum-free medium. The tissues were washed with PBS. Then tissues were incubated for 48 h in fresh culture medium. The tissues were then frozen in OCT, and X-Gal staining was used to measure the Ad-CMV-LacZ and Ad-LP-LacZ expression on the section slides.

Studies of *in Vitro* Vector-infected Ovarian Cancer Cell Line in Nude Mice

Ovcar-5 tumor cell lines were infected *in vitro* at 100 MOI with either the Ad-Lp-LacZ or Ad-Lp-CD adenoviral vectors for 60 min, washed with PBS, and then resuspended in PBS (4×10^7cells/1 ml PBS). Ten female nude mice 6–8 weeks of age (25–28 grams in weight), which were purchased from Cox, Inc., Cambridge, MA, were injected i.p. with 40 million Ovcar-5 ovarian carcinoma cells previously infected at 100 MOI with the Ad-Lp-LacZ vector. An additional 10 26–28-gram mice 6–8 weeks of age were injected i.p. with Ad-Lp-CD-infected cells. From the second day, all 20 of the mice were injected once a day with 5FC at 500 mg/kg i.p. for 10 days. Three weeks after tumor cell injection, the 10 Ad-Lp-LacZ-injected mice and 7 of the Ad-LP-CD-injected mice were killed and autopsied. At the 50th day, another three Ad-LP-CD-injected mice were killed and autopsied.

In other experiments, five female nude mice were injected i.p. with 40 million Skov-3 cells previously infected *in vitro* with the Ad-Lp-CD vector at 80 MOI. Another five mice were injected i.p. with Skov-3 cells previously infected *in vitro* at 80 MOI with the Ad-Lp-LacZ vectors. Then all of the 10 mice were injected i.p. with 500 mg/kg of 5FC daily for 10 days. Three weeks later, the mice were killed and autopsied (18, 19).

Studies of *in Vivo* Intratumoral Injection of Adenoviral Vectors

EJ cells (5×10^6) in PBS were injected s.c. in 25 nude mice. Three weeks later, the tumor size (width and length) was measured, then the tumor volume (mm^3) was calculated according to the formula: Tumor volume = length $\times$ width^2/2 (20, 21). Then, tumor nodules in eight mice were injected with 10^8 pfu of the Ad-CMV-CD virus. Tumor nodules in an additional eight mice were injected with 10^8 pfu of the Ad-Lp-CD virus, and tumor nodules in another nine mice were injected with 10^8 pfu of the Ad-CMV-LacZ virus. After this, 500 mg/kg of 5FC was injected into the peritoneal cavity each day, once a day, for 5 days. Two weeks later, we measured the tumor size again and compared the tumor growth before and after the treatment with viral particles and 5FC. Another 20 nude mice were injected s.c. with 5×10^6 Ovcar-5 tumor cells. After this, the same vector injections and 5FC treatments were conducted as for the EJ tumor cell in the nude mice. Autopsy of the mice was carried out, and H&E-stained sections of the tumor and the adjacent tissues were examined to measure the toxicity of the vectors.

RESULTS

Study of Factors Affecting Percentage of β-Galactosidase-positive Cells after Exposure to the Ad-CMV-LacZ or Ad-Lp-LacZ Vectors. The infectivity of cell lines by adenoviral vectors has been reported to be dependent on the presence of the CAR, which mediates the binding of the vector to the target cell (22–24), the level and functional state of both the αvβ3 and αvβ5 integrin receptors, which are important for endocytosis of the vector, and the release of the vector from the endosome (25–27). Cell lines in which the αvβ3 receptors are low or functionally inactive may have low levels of expression of vector transgenes, because the amount of vector DNA reaching the nucleus, where it is transcribed into mRNA, will be reduced in αvβ -deficient cell lines because of sequestration in the endosome.

To study the effect of these receptors on the uptake of the adenoviral vector into cancer cell lines and the subsequent expression of its *LacZ* transgene in target cells, the Ovcar-5, Hey, and Skov-3 ovarian cancer cell lines, the EJ, and J82 bladder cancer cell lines, and the CDD minimal deviation cancer cell line were exposed to the Ad-CMV-LacZ vector. Then these cell lines were studied for the percentage of cells that were positive for β-galactosidase. We chose a vector with the CMV promoter, because this promoter is known to be active in most, if not all, mammalian cells. Differences in β-galactosidase in these cell lines would therefore be attributable to differences in binding and endocytosis of the vector or release of the vector from the post-entry endosome. As shown in Table 1, the cells of all of the established ovarian and bladder cancer cell lines studied had a high percentage of cells positive for the CAR receptor (except for the Hey ovarian carcinoma cell line, in which none of the cells were detectable as positive for CAR). Among the established carcinoma cell lines in which a high percentage of cells were positive for CAR, all of the cell lines except for the Ovcar-5 cell line had >80% of the cells positive for the αvβ5 receptor. The percentage of Ovcar-5 cells positive for the α v β 5 integrin receptor was 57%. The percentage of cells positive for the αvβ3 integrin receptor was more variable among the cell lines. Only one-half of the Ovcar-5 cells were positive for the either of the integrin receptors.

Not surprisingly, a high percentage of the cells of all of the established tumor cell lines studied, except for the Hey cell line, were detectable as positive for β-galactosidase after exposure to the Ad-CMV-LacZ vector (see Table 1). This suggests that cell lines in which a high percentage of cells are positive for both the CAR and the αVβ5 integrin receptors will be infectible by the adenoviral vectors and therefore will score positive for the protein product of a vector transgene if the transcriptional promoter driving the expression of the transgene is very strong, as is the case with the CMV promoter. Surprisingly, as shown in Table 1, although only 30% of the cells of the CCD cell line are positive for the CAR receptor and only 63% of the CCD cells were positive for the αvβ3 integrin receptors, up to 70% of the CCD cells are positive for β-galactosidase after exposure to the Ad-CMV-LacZ vector. Experiments carried out previously in our laboratory have shown that the CCD cell line is infectible by the Ad-CMV-LacZ vector (7). This suggests that there may be a CAR-independent mechanism of binding of the adenoviral vector to the CCD cells, and that the strength of a transcriptional promoter may overcome in part the limitation imposed on transgene expression by a lower level of the αvβ3 receptor.

Comparison of *Lac-Z* Gene Expression Levels in Cell Lines Infected with Either the Ad-CMV-LacZ or the Ad-Lp-LacZ Vectors. Another factor that may alter the percentage of cells scoring positive for transgene expression after exposure to an adenoviral vector is the level of activity of the transcriptional promoter regulating the vector transgenes in these different cell lines. Because it had been reported that the *L-plastin* gene was detectable in most tumor cell lines, but not in any normal cells of the body except for the mature leukocyte (4, 6), the same cell lines exposed to the Ad-CMV-LacZ vector were also exposed to an adenoviral vector in which the *LacZ* gene was regulated by the L-plastin promoter (Ad-Lp-LacZ).

Table 1 *Characterization of percentage of cells positive for the CAR, αvβ3, and αvβ5 receptors as measured by FACS analysis and study of infectivity of cells by Ad-CMV-LacZ Vector at 20 MOI as measured by β-galactosidase assay (X-Gal)*

αvβ3, αvβ5, and CAR receptor levels were measured by mouse monoclonal antibodies, and the FITC-conjugated antimouse antibody was used to stain the cells. Then, FACS analysis was used to detect the percentage of the receptor-positive cells (n = 2). For infectivity, cells were exposed to virus in serum-free medium for 90 min at 20 MOI and incubated for 48 h in culture medium. Then, cells were stained by X-Gal analysis (n = 2).

	αvβ3	αvβ5	CAR	β-Gal
EJ	83 ± 8	82 ± 5	95 ± 8	95 ± 8
J82	56 ± 6	78 ± 7	80 ± 10	88 ± 10
Skov-3	64 ± 6	91 ± 8	87 ± 7	85 ± 11
Ovcar-5	48 ± 7	57 ± 5	88 ± 10	65 ± 8
Hey	81 ± 5	96 ± 10	0	10 ± 4
CCD	63 ± 8	93 ± 4	29 ± 5	70 ± 9

Table 2 *Comparison of β-galactosidase levels in cell line exposed to Ad-CMV-LacZ or Ad-Lp-LacZ (ONPG, OD)*

Cells were exposed in serum-free conditions for 90 min at 20 MOI. After 48 h of incubation in culture medium, the level of the β-galactosidase (ONPG) in each cell line was measured by optical density, as outlined in "Materials and Methods" ($n = 2$).

	Ad-CMV-LacZ	Ad-Lp-LacZ	Ratio of CMV/Lp
EJ	1.1 ± 0.2	0.9 ± 0.1	1.2
J82	1.0 ± 0.1	0.4 ± 0.1	2.5
Skov-3	0.9 ± 0.1	0.4 ± 0.1	2.2
Ovcar-5	0.9 ± 0.1	0.4 ± 0.1	2.2
CCD	0.9 ± 0.1	0.1 ± 0.01	9

To determine whether the L-plastin promoter was selectively more active in epithelial neoplastic (ovarian and bladder cancer) cell lines than in minimal deviation fibroblast cell line (CCD), we tested the *LacZ* gene expression levels in the Ovcar-5, EJ, J82, Skov-3, and CCD cell lines after exposure to either the Ad-CMV-LacZ or the Ad-Lp-LacZ vectors. We then calculated the ratio of β-galactosidase levels in cells infected with the Ad-CMV-LacZ, divided by the β-galactosidase levels in cells infected by the Ad-LP-LacZ vectors, as an index of the L-plastin promoter strength in established ovarian or bladder cancer cell lines as compared with the minimal deviation CCD fibroblast cell line.

As shown in Table 2, the amount of β-galactosidase in the EJ, J82, Skov-3, Ovcar-5, and CCD cell lines after exposure to the Ad-Lp-LacZ vector was less than for the same cells exposed to the Ad-CMV-LacZ vector. This suggested that the Lp promoter was less strong in all of the cell lines than the CMV promoter, thereby decreasing the percentage of cells that scored positive for the vector transgene protein product under any given level of integrin or CAR receptor representation or function. A comparison of the amount of β-galactosidase in each cell line after exposure to the Ad-CMV-LacZ was divided by that for the Ad-Lp-LacZ vector. This ratio was 2 in all of the established cancer cell lines except for the CCD cell line, in which the ratio was 9. One possible explanation for this difference was that the CCD cell line supported the expression of the Lp promoter to a much lesser extent than the CMV promoter.

The relatively low *LacZ* gene expression in the CCD cell line exposed to the Ad-Lp-LacZ vector is not attributable to the low infectivity by the Ad-Lp-LacZ vector, because, as shown in Tables 1 and 2 and in a previous publication from our laboratory (7), >70% of the CCD cells were positive for β-galactosidase after exposure of these cells to the Ad-CMV-LacZ vector, indicating that the CCD cells are infectible by adenoviral vectors. The ratio of β-galactosidase levels in Ad-CMV-LacZ-infected cells divided by the β-galactosidase levels in Ad-Lp-LacZ-infected cells was much higher in CCD than in cell lines derived from bladder cancer and ovarian cancer. These data suggest that the L-plastin promoter is much more active in epithelial neoplastic cell lines than in the CCD minimal deviation fibroblast cell line.

Studies of the Effect of 5FU Released from Infected Cells on Noninfected Cells. To monitor the effect of 5FU released from infected cells on the noninfected cells, mixtures of Ad-CMV-CD or Ad-Lp-CD vector-infected and -noninfected cells were generated and then exposed to 5FC. The CD protein converts the nontoxic prodrug 5FC into the toxic chemical 5FU. Unphosphorylated 5FU can be released from cells infected with the CD vector and taken up by surrounding uninfected cells and can kill the uninfected cells. This is called the bystander effect. As shown in Fig. 1, when as few as 5% of the population of Ovcar-5 cell lines or the CCD minimum deviation fibroblast cell line infected with Ad-CMV-CD (160 MOI) vectors were mixed with 95% of uninfected cells, the majority of the cells were killed when cells were exposed for 5 days to 5FC at a 500 μM concentration (18% Ovcar-5 and 29% CCD cells survived). This suggests that only a few of these cells need to be infected with the Ad-CMV-CD adenoviral vector to generate sufficient levels of 5FU *in vivo* in static cell culture to kill the vast majority of infected as well as uninfected tumor cells. The high percentage of cells killed at low infectivity *in vitro* is attributable partly to the fact that the medium was not changed, and therefore the cells were exposed continuously to a high level of 5FU, which continues to increase with time. In these conditions, the high levels of 5FU released from a few Ad-CMV-CD vector-infected cells could kill all of the uninfected cells.

When the cell lines were infected with the Ad-Lp-CD vector, incomplete cell death was seen even at the highest MOI tested with the CCD human minimal deviation cell line. In contrast, almost all of the cells were eradicated at the highest (160) MOI when similar experiments were carried out with the Ad-Lp-CD vector in the Ovcar-5 cell line (see the data in Fig. 1, *A* and *C*, 160 MOI). The

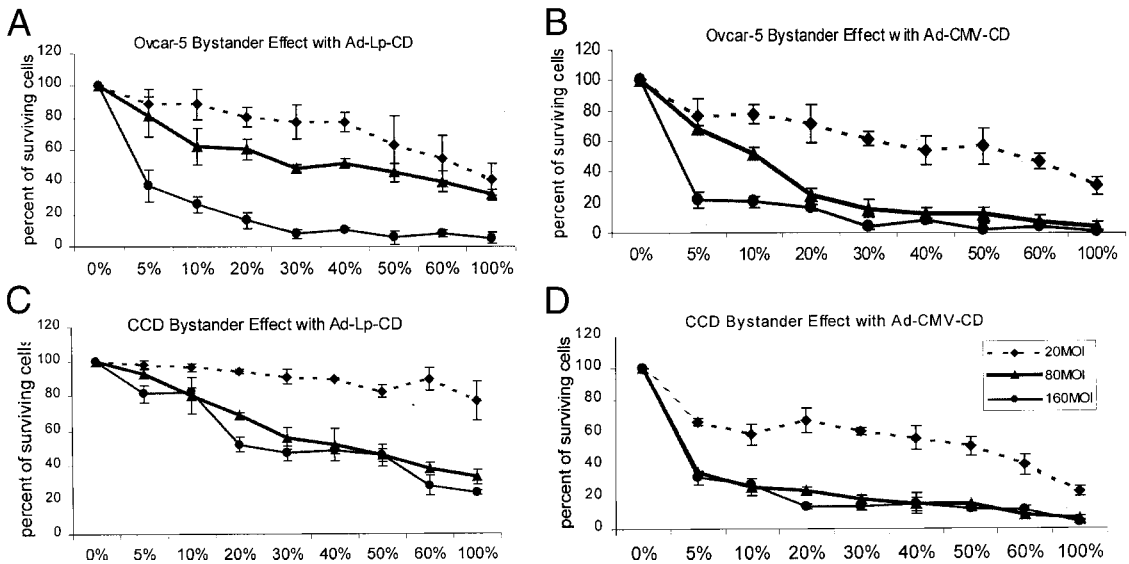

Fig. 1. Toxicity of vectors at varying levels of infected cells. Ovcar-5 (*A* and *B*) or CCD (*C* and *D*) cell lines were infected at varying MOI (20, 80, and 160) using the Ad-CMV-CD (*B* and *D*) or Ad-LP-CD (*A* and *C*) adenoviral vectors. The infected cells and noninfected cells were mixed in varying ratios to generate 0, 5, 10, 20, 30, 40, 50, 60, and 100% infected cells. Then cells were seeded in six-well plates and incubated for 5 days in 500 μM 5FC. Then the cells were trypsinized, and surviving cells were counted by trypan blue exclusion.

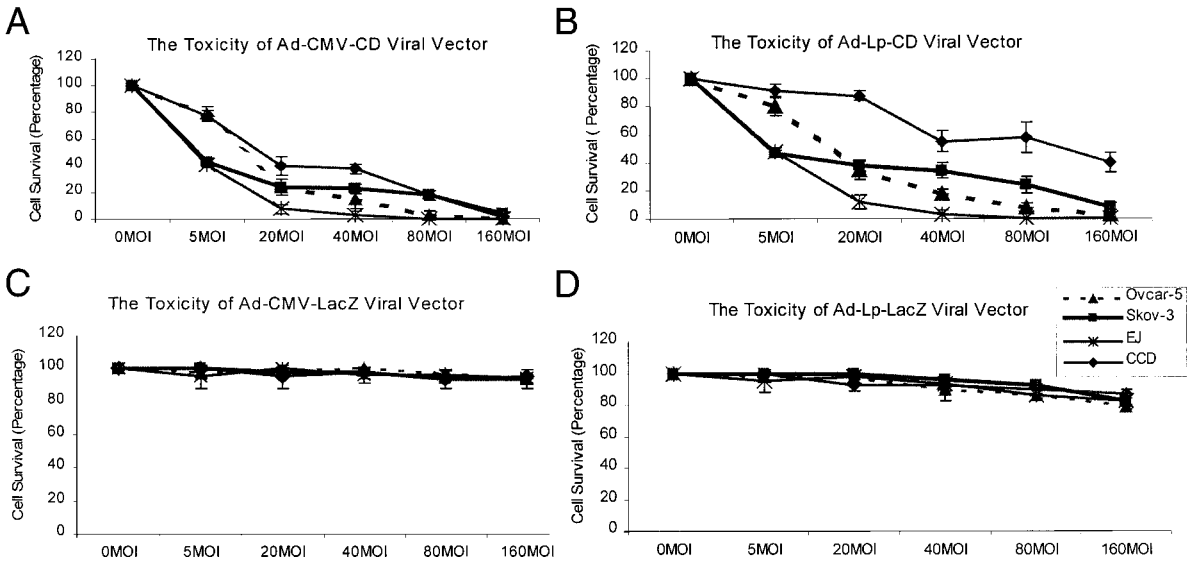

Fig. 2. Study of the toxicity of the control Lac-Z vector *versus* the CD vector. Cells (2×10^5) were infected at 0, 5, 20, 40, 80 and 160 MOI of vector with Ad-CMV-CD (*A*), Ad-Lp-CD (*B*), Ad-CMV-LacZ (*C*), Ad-Lp-LacZ (*D*) vectors for 90 min. Then cells were seeded in six-well plates in duplicate and incubated in 500 μM 5FC for 5 days. The percentage of surviving cells was counted by trypan blue exclusion.

difference in the survival of cells between the CCD and Ovcar-5 cells when exposed to the Ad-Lp-CD vector and 5FC was statistically significantly different at the $P < 0.001$ level. (An analysis of variance was used to determine whether the percentage of surviving cells was statistically significantly different when the Ad-CMV-CD or Ad-Lp-CD vectors were used to infect either the CCD or the Ovcar cell lines.) This difference could be attributable to differences in the infectivity of the CCD cell line, the sensitivity of this cell line to 5FU, or a difference in the expression of the Lp-driven CD transcription units in the Ovcar-5 or CCD cell lines. As shown in Fig. 1*D*, when the CCD cell line was exposed to 160 MOI of the Ad-CMV-CD vector and 5FC, complete killing of the CCD cells occurred at 100% infection. There were no differences between the cell kill in the Ovcar-5 and CCD cell lines with the Ad-CMV-CD vector (compare the survival data at 160 MOI at 100% infection in Fig. 1, *B* and *D*). Therefore, the differences seen in *A* and *C* in the cell survival of the Ovcar-5 and CCD cell lines after exposure to the Ad-Lp-CD vector are not attributable to differences between the CCD and Ovcar-5 cell lines with respect to infectivity by the virus or sensitivity to 5FU, because complete killing is seen with the Ad-CMV-CD vector with the CCD cell line.

This suggests that the L-plastin promoter is less active in the CCD minimal deviation fibroblast cell line than in the established tumor cell line Ovcar-5. The high levels of cell-killing at low infectivity that were seen *in vitro* with the Ad-Lp-CD vector will probably not be seen *in vivo* because of removal of the 5FU by blood flow and metabolic degradation.

5FU Sensitivity of Each Cell Line Expressed as IC_{50}. It is possible that the low-level cell death of the CCD cell line could be attributable to intrinsic resistance to 5FU toxicity, which is greater than that seen in the Ovcar-5 or other established cancer cell lines. To test this, the intrinsic sensitivity of each cell line to 5FU was measured by seeding 3×10^5 cells in T25 flasks in triplicate, which were incubated for 96 h at different 5FU concentrations. The IC_{50} generated for 5FU in the J82 cell line is 55 μM, for the EJ cell line is 30 μM, for the Ovcar-5 cell line is 3 μM, for the Skov-3 cell line is 22 μM, and for the CCD cell line is 15 μM. The IC_{50} generated for 5FU in the CCD cell line (15 μM) is less than that of several of the epithelial neoplastic cell lines (EJ, J82, and Skov-3), suggesting that the CCD cell line is as sensitive to 5FU as the epithelial cancer cell lines. Thus, the low sensitivity of the CCD fibroblast cell line to the effect of the Ad-Lp-CD vector/5FC treatment is not attributable to a high level of resistance to 5FU, but rather to low levels of the protein product of the transcription units driven by the L-plastin promoter in the CCD cell line.

Study of 5FC Toxicity of the Adenoviral Vectors Carrying the CD Transcription Units. To test how much of the toxicity of the Ad-Lp-CD/5FU treatment was attributable to the toxicity of the vector backbone and how much was due to the protein produced by the CD transcription unit, the cell lines were infected with the Ad-CMV-CD, Ad-CMV-LacZ, Ad-Lp-CD, or Ad-Lp-LacZ vectors at different MOI. After this, the cell lines were incubated in medium supplemented with 500 μM 5FC for 5 days. As shown in Fig. 2, no significant toxicity was seen with any of the cell lines when the backbone vector, Ad-CMV-LacZ, and Ad-Lp-LacZ were used (see Fig. 2, *C* and *D*). In contrast, when the cell lines were exposed to the Ad-Lp-CD or the Ad-CMV-CD vectors, nearly 100% killing of the cell lines after exposure to the vector and to 5FC was seen in all cell lines with both vectors, with the exception of the example of the CCD cell line after exposure to the Ad-Lp-CD vector and 5FC. The cell killing for CCD after exposure to the Ad-Lp-CD *versus* the Ad-CMV-CD vectors and 5FC (see Fig. 2, *A* and *B*) is statistically significantly different at the $P < 0.01$ level by the *t* test. No statistically significant differences were seen in any of the established tumor cell lines with respect to cell survival. This indicates that the toxicity seen in Fig. 2 after exposure to the Ad-Lp-CD or Ad-CMV-CD vectors is not attributable to the adenoviral backbone but to the action of the CD protein and 5FC. The <100% cell kill in the example of the CCD after exposure to the Ad-Lp-CD vector and 5FC is most probably attributable to the lower level of transcriptional activation of the *CD* gene by the Lp *versus* the CMV promoter, as explained above. Thus, the toxicity seen in these experiments was not attributable to the viral backbone, but to the effect of the CD transcription units on the conversion of 5FC to 5FU. In addition, the 5FC toxicity generated by incubation of the Ad-Lp-CD transcription units in bladder cancer or ovarian cancer cell lines is statistically significantly higher than that seen in the CCD cell line.

Table 3 *Percentage of cells in explant cultures of ovarian cancer cells and normal peritoneal cells which score positive for β-galactosidase after exposure to the Ad-CMV-LacZ and the Ad-Lp-LacZ vectors*

Samples of primary tumor, metastatic tumor, and normal peritoneum were cut into small pieces. These pieces were then digested with collagenase to produce tissue disaggregation, and the resulting cells were cultured in RPMI 1640 with 10% NBCS. All experiments were performed at 90% confluence. Samples of ascites were divided into the T25 flasks directly and washed to remove debris after cell attachment. Cells were infected in the flasks for 90 min, and after 48 h of incubation, the positive cells were measured by X-Gal staining or FACS.

		Ascites	Primary tumor	Metastatic tumor	Normal peritoneum
Ad-CMV-βgal	*X-Gal*	50–80%	50–90%	45–85%	60–80%
	FACS	95%	94%	94%	
Ad-LP-βgal	*X-Gal*	10–35%	15–60%	15–45%	1–4%
	FACS	39%	83%	38%	
CMV/LP ratio	*FACS*	3/1	1/1	3/1	20–60/1

Percentage of Cells Detectable Positive for LacZ Expression in Primary Monolayer Cultures of Samples Obtained at Surgery from Normal Peritoneum and Metastatic Implants of Ovarian Cancer after Exposure to the Ad-Lp-LacZ or Ad-CMV-LacZ Vectors. Samples of metastatic tumor and normal peritoneum were collected from 16 ovarian cancer patients undergoing diagnostic or therapeutic laparotomy. The tumor was cut into small pieces and then digested with collagenase to disaggregate the tissue. The resulting cells were then cultured in RPMI 1640 supplemented with 10% NBCS. After culture, the cells were exposed at a MOI of 20 to the Ad-CMV-LacZ or Ad-Lp-LacZ vectors in T-flasks for 90 min. After 48 h of incubation, the percentage of β-galactosidase-positive cells was measured by X-Gal staining or FACS (28). A ratio of β-galactosidase-positive cells with the two vectors was generated by dividing the percentage of cells that were detectable as positive for β-galactosidase by FACS after exposure to the Ad-CMV-LacZ vector by the percentage of cells detectable as positive for β-galactosidase by FACS after exposure to the Ad-Lp-LacZ vector. As shown in Table 3, this ratio was at least 20–60-fold higher in the normal peritoneal cells than with any of the samples derived from ovarian cancer cells. These results indicate that the normal peritoneal cells are less able to support the expression of transgenes driven by the L-plastin promoter than are the ovarian cancer cells.

Table 4 *Cytotoxicity in monolayer culture of normal peritoneum and ovarian cancer cells after expression to Ad-Lp-CD and Ad-CMV-CD vectors and 5FC (percentage of cells killed)*

In Ad-CMV-CD- and Ad-Lp-CD-infected samples, 500 μM 5-FC were added and incubated for 5 days, then the percentage of cells killed was estimated by comparing the percentage of cells which had died in the infected and uninfected control flasks.

	Ad-CMV-CD	Ad-Lp-CD
Ascites	98%	85%
Metastatic tumor	85%	70%
Primary tumor	90%	75%
Normal peritoneum	95%	10%

Cytotoxicity after Exposure of the Monolayer Cell Cultures of Normal Peritoneum and Ovarian Cancer from Surgical Specimens to the Ad-CMV-CD and Ad-Lp-CD Vectors. Samples of primary tumor, metastatic tumor, and normal peritoneum were collected from 16 ovarian cancer patients, and samples were prepared by the same methods as described previously. As shown in Table 4, when the cells were infected with the CD vectors and incubated for 5 days in the presence of 500 μM 5FC in T25 flasks, the majority of the cells in the explant cultures of primary ovarian cancer, metastatic ovarian cancer, and ovarian cancer in ascites were killed by the Ad-CMV-CD or the Ad-Lp-CD vectors and 5FC. In contrast with the results in the ovarian cancer cells, in which the cell death with the Ad-CMV-CD and Ad-Lp-CD vectors was roughly the same, in the case of the biopsies of normal peritoneum, the cell death with the Ad-Lp-CD vector was only one-tenth of that seen with the Ad-CMV-CD vector. This indicates that the expression of the L-plastin promoter-driven *CD* gene is much lower in the peritoneum than in the ovarian cancer cells.

Studies of LacZ Vectors in Organ Cultures of Normal Ovary. Samples of ovarian cancer and normal ovary tissues were cut into small pieces then inoculated in organ culture for 24–48 h and infected with either the Ad-CMV-LacZ or the Ad-LP-LacZ vectors for 90 min. Then fresh medium was added, and the tissues were incubated for 48 h and then processed to the slide sections for study by the X-Gal staining reaction. The organ culture differs from the monolayer culture in that the organ culture is a three-dimensional array of cells. As shown in Fig. 3, there is a much stronger blue staining in the outer edges of the cell mass in the organ cultures of normal ovarian tissue with the Ad-CMV-LacZ vector, middle panel, than with the Ad-Lp-LacZ vector, right-hand panel. The results indicate that the CMV promoter is much more active in normal ovarian tissue than is the L-plastin promoter.

Killing Efficiency of Ovarian Cancer Tumor Cell Lines by 5FC/CD Vector System in Nude Mice. To test the efficacy of the Ad-Lp-CD replication incompetent vector system in a mouse human

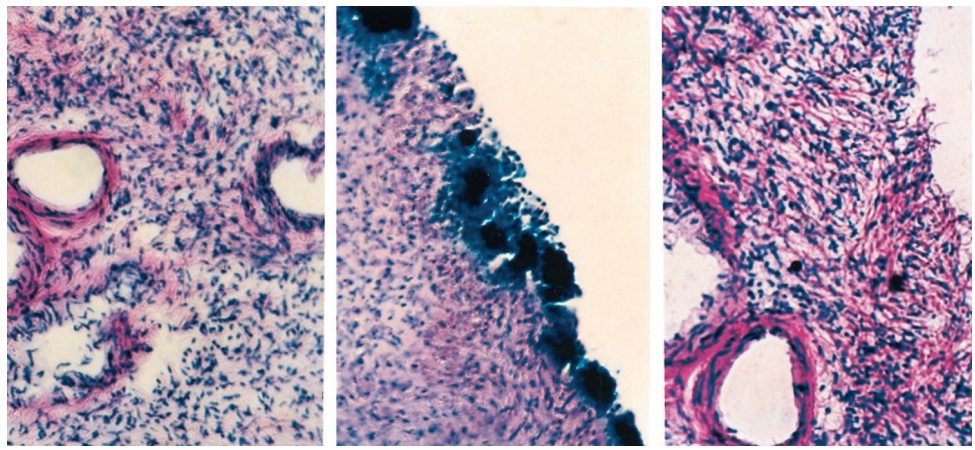

Fig. 3. Ovarian organ cultures. Normal ovarian tissue was obtained from patients undergoing abdominal surgical procedures. The tissues were cut into small pieces and cultured in DMEM:Ham's F12 medium with 10% charcoal-stripped serum. Twenty-four to forty-eight h later, the tissues were infected with vectors for 90 min, washed with PBS, and then incubated for 48 h. Then the tissues were frozen in OCT and sectioned, after which the frozen sections were stained by the X-Gal reaction. *Left*, no vector; *middle*, Ad-CMV-LacZ vector; *right*, Ad-Lp-LacZ vector.

Table 5 *Tumor growth in animals injected with adenoviral LP vectors (percentage of animals found to be positive for tumors)*

The SCID mice were injected with 40 million Ovcar-5 or Skov3 tumor cells, which had been infected previously *in vitro* with the Ad-Lp-LacZ vector or the Ad-Lp-CD vector. Starting on the second day, 500 mg/kg 5-FC was injected each day for 10 days. Animals were autopsied at 21 days after tumor cell injection, and the presence or absence of tumor nodules in the peritoneal cavity was assessed.

	Ad-Lp-LacZ-infected	Ad-Lp-CD-infected
Ovcar-5 (100 MOI)	10/10 (100%)	0/10 (0%)
Skov3 (80 MOI)	5/5 (100%)	0/5 (0%)

tumor xenograft model, we first exposed the Skov-3 ovarian cancer cell line to the Ad-Lp-CD vector *in vitro* at 80 MOI or the Ovcar-5 ovarian cancer cell line to the Ad-Lp-CD vector *in vitro* at 100 M0I by incubating the cells in the vector for 60 min. Then we injected 40 million of these *in vitro*-infected Ovcar-5 vector infected cells into 10 nude mice or injected the *in vitro*-infected Skov-3 ovarian carcinoma cell line into 5 nude mice. One day after injecting the tumor cells, we initiated daily i.p. injections of 5FC into each of the animals to generate a daily peak of i.p. 5FC concentrations in the 500-μM range. We carried on the daily i.p. 5FC injections for 10 days after the tumor injection. At 21 days after injection into the mice, we killed seven of the Ovcar-5-injected mice and all five of the Skov3-injected mice and examined the peritoneal cavity for tumors. The remaining three Ovcar-5 mice that were not killed at 21 days were killed at 50 days after tumor injection. As shown in Table 5, all of these animals were free of detectable tumor nodules, either at the gross morphological level or at the histopathological level.

In contrast, as shown in Table 5, all of the animals injected with the Ovcar-5 *in vitro*-infected cell lines and all five of the animals injected with the Skov-3 *in vitro* tumor cells previously infected *in vitro* with the control Ad-Lp-LacZ virus had detectable signs of tumor cell growth, either at the gross level or at the microscopic level. These data show that, in principal, it is possible to prevent engraftment of tumor cells in nude mice if all of the tumor cells are infected *in vitro* before i.p. injection of the cells with the replication-incompetent Ad-Lp-CD vector, and the animals are injected on a daily basis with the prodrug (5FC) which is converted into 5FU in the tumor cells.

To test the effect of administering the replication-incompetent Ad-Lp-CD and the Ad-CMV-CD vectors *in vivo* to preexisting s.c. nodules, we also tested the effect of intratumoral *in vivo* injection of established tumor nodules with the LacZ control vector, the CMV-CD vector, and Lp-CD adenoviral vector on the growth of the s.c. tumor nodules. As shown in Fig. 4, the tumors injected with the control Ad-CMV-LacZ vectors increased 3–4-fold after vector injection. In contrast, the size of both the Ovcar-5 and the EJ cell tumor nodules injected with the Ad-CMV-CD or the Ad-Lp-CD vector was one-third to one-sixth of the size of the tumors injected with the Ad-CMV-LacZ vector. The growth of the Ovcar-5 or EJ cancer cell lines after exposure to the Ad-CMV-LacZ vector was statistically significantly greater than the growth of the Ovcar-5 or EJ cell lines after exposure to either the Ad-CMV-CD or the Ad-Lp-CD vectors, at the $P < 0.001$ level, by the t test of the ratios (two-tailed). There was no statistically significant difference in the growth of the Ovcar-5 or the EJ cancer cell lines exposed to the Ad-CMV-CD *versus* the AD-Lp-CD vectors.

To determine whether there was toxicity to the normal tissues, we studied histopathological sections of the tumor nodules and surrounding normal tissues by light microscopic examination after injection with the Ad-Lp-CD, Ad-CMV-CD, or Ad-CMV-LacZ vectors after exposure to 5FC. As seen in Fig. 5, *in vivo* injection of the Ad-Lp-CD

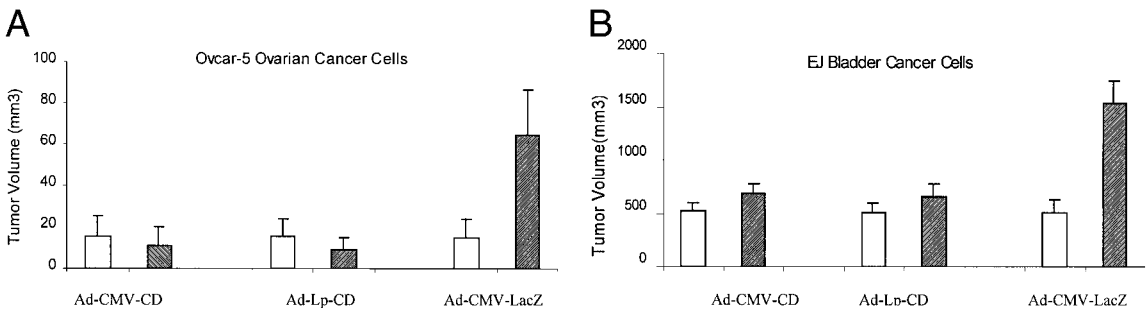

Fig. 4. Effect of *in vivo* injection of tumor nodules with adenoviral vectors. Ovcar-5 (*A*) or EJ (*B*) cells (5×10^6) were injected s.c. into nude mice. After 3 weeks, the tumor nodules were measured. Then, 10^8 pfu of the Ad-CMV-CD, 10^8 pfu of the Ad-Lp-CD, or 10^8 pfu of the Ad-CMV-LacZ vectors were injected into each tumor nodule, and 500 mg/kg of 5FC was given i.p. once a day for 5 days. Seven days later, the tumor nodules were measured again. □ shows tumor volume before viral particles and 5FC treatment; ■ shows the tumor volume 7 days after exposure to viral particles and 5FC treatment.

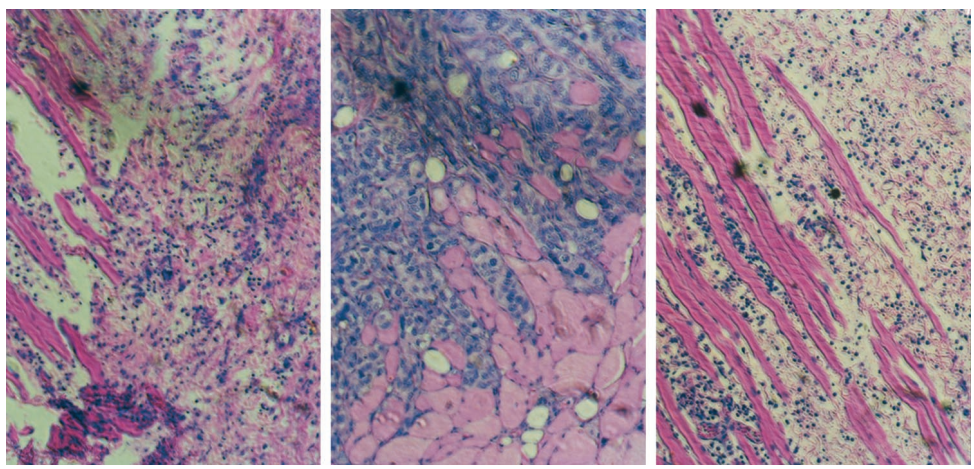

Fig. 5. Vector toxicity to tumor cells and adjacent tissues. Ovcar-5 cells ($5x10^6$) were injected s.c. After 3 weeks, 10^8 pfu of Ad-Lp-CD (*right*), 10^8 pfu of Ad-CMV-CD (*left*), or 10^8 pfu of Ad-CMV-LacZ (*middle*) vector were injected into each tumor nodule, and 500 mg/kg 5FC was given i.p. once a day for 5 days. *Right* (Ad-Lp-CD), most of the tumor cells are necrotic, whereas the adjacent muscle cells have a normal structure. *Left* (Ad-CMV-CD), after injection with the Ad-CMV-CD vector, the tumor cells are necrotic. *Middle* (Ad-CMV-LacZ), after injection of the Ad-CMV-LacZ vector, neither the muscle nor the tumor are necrotic.

vectors into the tumor nodules generated toxicity to the tumor cells (*right*). In Ad-CMV-CD *in vivo*-injected tumors, the tumor cells underwent necrosis (*left*). The toxicity to the tumor with the Ad-Lp-CD vectors was every bit as extensive in the tumor as that seen with the Ad-CMV-CD vector. This data shows that the toxic effect of the Ad-Lp-CD vector/5FC system is as great as that generated by the Ad-CMV-CD/5FC system, and the toxic effect of these two vectors is much greater than that seen with the Ad-CMV-LacZ vector.

DISCUSSION

A major limitation of the existing adenoviral vectors used for cancer gene therapy is the nonselective toxic action of these vectors. Attempts to render these vectors more selective for tumor cells and less toxic for normal cells has involved the use of tissue-specific transcriptional promoters to drive the therapeutic transcription units for these vectors. One of the limitations that have characterized these tissue-specific promoters is that the vectors carrying these tissue-specific therapeutic transcription units are usually less robust in their antitumor toxic action than nonselective viral transcriptional promoters.

We have reported the use of a tumor-specific rather than a tissue-specific transcriptional promoter for the regulation of an adenoviral therapeutic transcription unit. The L-plastin promoter was chosen because no normal tissue except for the mature leukocyte exhibits expression of the *L-plastin* gene. In contrast, most of the established cancer cell lines exhibit high levels of the expression of this gene. Experimental results published previously by our laboratory (7) have shown that a truncated L-plastin promoter retained its high activity within ovarian cancer cells, whereas it was relatively inactive in explants of normal peritoneal lining mesothelial cells. This data suggested that adenoviral vectors carrying therapeutic transcription units regulated by the L-plastin promoter might be useful in treating ovarian cancer.

When the L-plastin promoter is used to drive the expression of CD chemotherapy sensitization transcription unit in static cultures *in vitro*, only 50% of the cancer cells need to be infected to kill 100% of the epithelial neoplastic cells. In contrast, the percentage of cells that die in populations of CCD fibroblast cells is much lower, never reaching 100%. At all MOI tested, there are statistically significantly different levels of cell death generated by exposure to the Ad-CMV-CD *versus* the Ad-Lp-CD vectors and 5FC ($P < 0.001$), presumably because of the lower levels of activity of the L-plastin promoter in the CCD cell line (see Fig. 1 and 2). Control experiments have shown that the CCD cell line is as infectible by the Ad-CMV-LacZ as are the epithelial cancer cell lines (see Table 1). This indicates that low infectivity is not responsible for the low sensitivity of the CCD cell line to the Ad-Lp-CD vector. In addition, the intrinsic sensitivity of the CCD to 5FU toxicity directly added to the culture is not lower than that seen in the epithelial neoplastic cell lines. Thus, it appears that the level of expression of the L-plastin-driven genes in the CCD cell line is lower than that seen in the ovarian and bladder cancer cell lines and this is responsible for the differential effect of the Ad-Lp-CD and Ad-CMV-CD vectors in the CCD *versus* the epithelial neoplastic cell lines.

Studies in primary normal mesothelial cells and primary cell cultures of ovarian cancer cells show that the ratio of cytotoxicity with CMV-driven CD adenoviral vectors: to Lp-driven CD adenoviral vectors is highest in normal peritoneum (ratio of 20–60) as compared with 3–5 times in ovarian cancer cells in malignant ascites or in primary or metastatic ovarian cancer. The use of the Ad-Lp-CD vector to infect ovarian cancer cell lines *in vitro* before their injection into the i.p. cavity of 5FC-injected nude mice results in a suppression of the engraftment of these ovarian cancer cells, whereas no sign of suppression of tumor growth occurred when the ovarian cancer cell lines were infected with the Ad-Lp-LacZ or Ad-CMV-LacZ control vectors.

These data suggest that, in principle, the L-plastin-regulated CD transcription units may selectively sensitize ovarian cancer cell lines to the effects of 5FC without significantly sensitizing the normal peritoneal surface cells to the effects of this 5FC/Vector system.

Many obstacles that remain to be overcome are pointed up by this data. The first is that, for such vectors to work *in vivo* in patients, some method must be developed for conferring conditional replication competency on these Lp-CD vectors so that they may infect 100% of the tumor cells when administered to patients with existing tumor *in vivo*.

The data in Fig. 3 shows that the expression of the reporter gene is seen only on the surface of an organ culture of cells infected with a replication-incompetent vector. Therefore, our laboratory is studying, on a preclinical level, several different types of adenoviral vectors that exhibit replication competency that is selective for the regulatory environment of the tumor cell. Our design is to use the L-plastin promoter to drive the expression of the adenoviral *E1A* gene, which is necessary for viral replication, as well as the CD chemotherapy sensitization gene. Such vectors may be useful in the i.p. therapy of ovarian cancer.

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The Use of the l-Plastin Promoter for Adenoviral-mediated, Tumor-specific Gene Expression in Ovarian and Bladder Cancer Cell Lines

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