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Award Number: DAMD17-99-1-9457

TITLE: Tumor Specific Gene Expression and Tumor Specific Vector Replication for Systemic Chemotherapy Sensitization Treatment of Breast Cancer

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REPORT DATE: July 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual (1 Jul 1999 - 30 Jun 2003)

4. TITLE AND SUBTITLE
Tumor Specific Gene Expression and Tumor Specific Vector Replication for Systemic Chemotherapy Sensitization Treatment of Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9457

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
We have developed a safety modified adenoviral vector in which the tumor specific L-plastin promoter can be used to control the expression of therapeutic genes such as cytosine deaminase (CD). The CD gene product catalyzes the conversion of a non-toxic precursor, 5-Fluorocytosine (5FC) into a drug, 5-Fluorocytosine (5FC), thereby sensitizing the cells in which it is produced to 5FC induced toxicity. We have also generated vectors in which the adenovirus needs to replicate within mammalian cells. The replication of a virus within a mammalian cell takes over all of the metabolic and cell proliferative machinery of the cells and thereby kills it. Our data shows that the adenoviral vector which carries the L-plastin-E1A transcription unit can replicate within breast cancer cells and other estrogen dependent carcinomas, such as ovarian cancer, while not being able to replicate in normal cells. This L-plastin-E1A vector can suppress the growth of human breast cancer cell lines in immunosuppressed mice. We have recently isolated a vector in which both the CD E1A genes are under the control of the L-plastin promoter, which is currently under study.

14. SUBJECT TERMS
Adenoviral vector, breast cancer, cytosine deaminase, 5FU

15. NUMBER OF PAGES
79

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction: This report summarizes progress on a project funded by the US Army Breast Cancer Research Fund (DAMD 17-00-9457). During the past year, one new manuscript has been published in Cancer Gene Therapy by Akbulut (which describes the performance of a double transcription unit vector for chemotherapy sensitization and E1A dependent tumor specific killing of cancer cells) and another manuscript which has been completed on the use of this same vector the development of a new chemotherapy regimen for colon cancer called "Genetic Saltz Therapy". This latter treatment regimen was shown to produce an advantage in terms of survival, remission duration and toxicity over the conventional chemotherapy program from which it was derived.

This progress was made possible by the first two years of the funding period in which a series of single transcription unit adenoviral vectors containing either the cytosine deaminase (CD) chemotherapy sensitization gene, or the adenoviral vector E1A viral replication gene. Three manuscripts and two reviews were published on the basis of this work. During the past year, a paper describing the first testing of a bicistronic vector in which the tumor specific L-plastin promoter was driving a bicistronic transcription unit composed of the CD gene and the E1A gene.

The end result of the 3 years of work is that a vector has been developed that can improve the performance of one of the most popular programs of chemotherapy for colon cancer.

Body: Narrative Description of the Results During the Reporting Period of Funding: July 1, 2000-June 30, 2003

I. Study of the Tumor Selectivity of L-Plastin Driven Transcription Units in Adenoviral Vectors

Injae Chung from our laboratory was the first to show that a truncated L-plastin promoter could produce high expression of transgenes in a tumor specific manner when placed in the adenoviral vector backbone. Chung showed (Cancer Gene Therapy 6: 99-106, 1999) that infection of explants of ovarian cancer cells with the AdVLpLacZ resulted in high expression levels of beta-galactosidase, whereas no LacZ gene expression occurred in explants of normal peritoneal epithelial cells (see Figure 1 below).

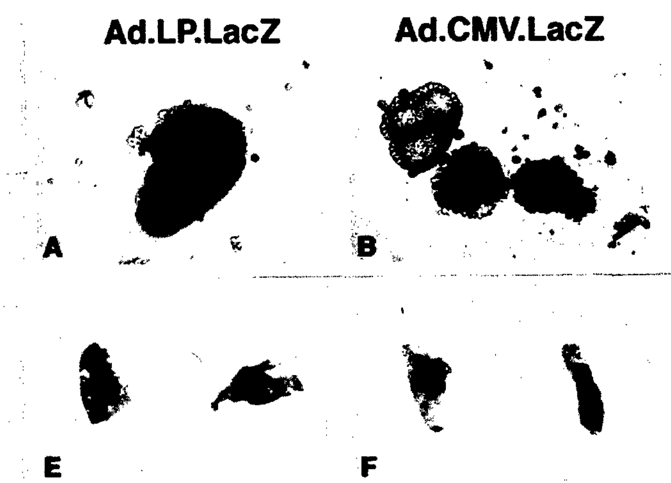


Figure 1 Panel A and B: Ascitic ovarian cancer cells infected with either the AD.LP.LacZ or the AD.CMV.LacZ vector. Panel E and F: Biopsy cells from the mesothelium of patients undergoing surgical procedures exposed to either the AD.LP.LacZ or the AD.CMV.LacZ vector.

XY Peng in our laboratory then showed (Cancer Research 61: 4405-4413, 2001)) that AdV with the LacZ reporter gene under the control of the L-plastin promoter (AdLpLacZ) produced beta-galactosidase activity in breast and ovarian cancer cell lines and explants of ovarian cancer but not in infected explants of normal peritoneal cells nor in organ cultures of normal ovarian epithelial cells.

II. Study of Vectors with L-Plastin Driven Cytosine Deaminase Transcription Unit.

She showed that there was much more toxicity of the AdLpCD vector when used in vitro with 5FC to explants of ovarian cancer than there was to explants of normal peritoneal tissue. In addition, the use of a replication incompetent AdV carrying a cytosine deaminase transcription unit under the control of the L-plastin promoter prevented engraftment of the Ovcar-5 and Skvo3 human ovarian cancer cell lines in the peritoneal cavity of nude mice.

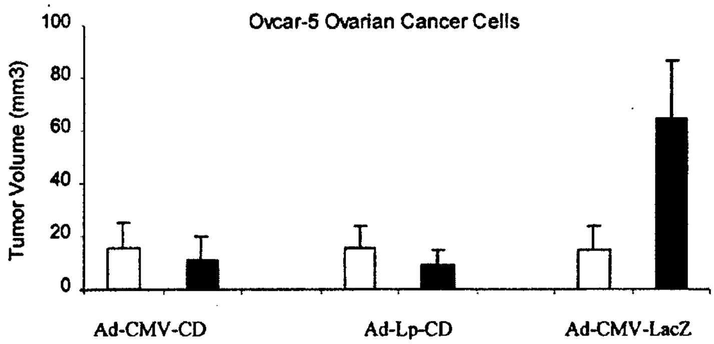


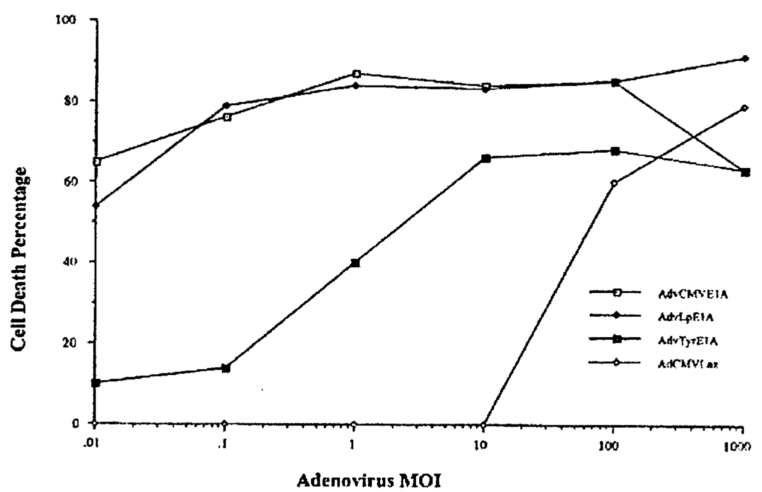
Fig. 2 Effect of *in vivo* injection of tumor nodules with adenoviral vectors.

There was no toxicity to the normal cells of the peritoneum. Finally, intratumoral injection of the AdLpCD vector suppressed the growth of ovarian cancer tumor nodules (see Figure 2).

III. Studies with L-Plastin Driven E1A Adenoviral Vectors.

Lixin Zhang of our laboratory created a series of AdV carrying the E1A gene under the control of the L-plastin promoter (AdVLpE1A). His experiments, which have been summarized in the Journal of Molecular Therapy (Mol. Therapy. 6: 386-393, 2002), showed that the AdVLpE1A vector was 1000 times more toxic to explant cultures of ovarian carcinoma than a vector carrying a LacZ transcription unit (see Figure 3 below and Figure 4 below).

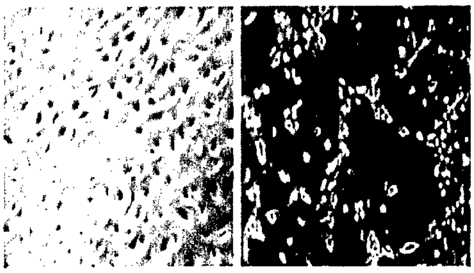
Figure 3



This AdLpE1A was as toxic to explants of ovarian cancer (see Figure 3) and as was the AdCMVE1A vector (see Figure 3). The AdLpE1A was not toxic to explants of normal breast epithelial cells (see Figure 5 below) whereas both the AdCMVE1A vector and the wild type adenovirus were toxic. This showed that Zhang's cytolytic vectors with the L-plastin promoter were tumor specific. Finally Zhang showed that the AdLpE1A suppressed human tumor xenografts of human breast cancer cell lines (both MCF-7 and MBAMD468) in a SCID mice (see Figure 6 below).

Figure 4

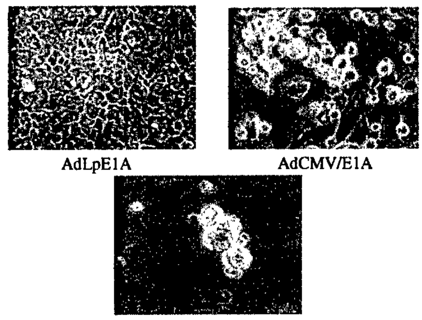
Ovarian Cancer Explants



No Vector AdLpE1AVector (1 MOI)

Figure 5

Explants of Normal Mammary Epithelial Cells



Wild Type Virus

IV. Vectors with L-Plastin Promoter Driven Bicistronic Transcripiton Units

The next step in the development of the AdV in the Deisseroth laboratory was the construction by Lixin Zhang of an AdV in which the L-plastin promoter regulated a bicistronic transcription unit composed of the cytosine deaminase gene linked to the E1A gene by an IRES element. These vectors were tested by Hakan Akbulut of our laboratory in collaboration with Lixin Zhang. The AdVLpCDIRESE1A vector was first compared in vitro with the AdVLpE1A vector and the AdVLpCD vector. The AdVLpCDIRESE1A was the most effective vector against the colonic cancer cell lines and much more effective than the single transcription unit vectors. Then, Abkulut studied intratumoral injection of the AdVLpCDIRESE1A with and without 5FC as compared to the AdLpCD with and without 5FC (Akbulut et al: The efficiency of replication-competent adenoviral vectors carrying L-plastin promoted cytosine deaminase gene in colon cancer)). Again, the intratumoral injection of the bicistronic vector with 5FC was much more effective in suppressing the growth of the human tumor xenograft than was the single transcription unit vector (see Figure 7 to the left). The work proposed in the research grant application is designed to complete the final step in the development of the adenoviral vectors for use as intracavitary therapy for ovarian cancer by modifying the fibrillar protein to target the adenoviral infection to ovarian cancer cells.

Figure 6

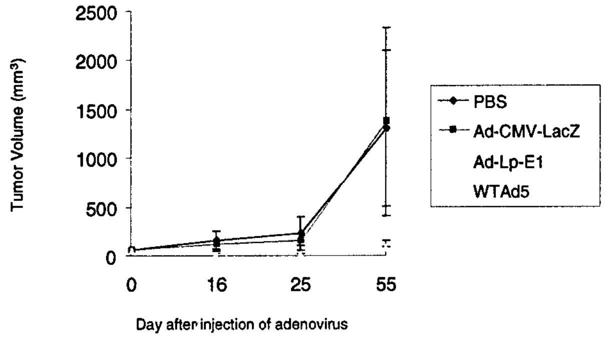
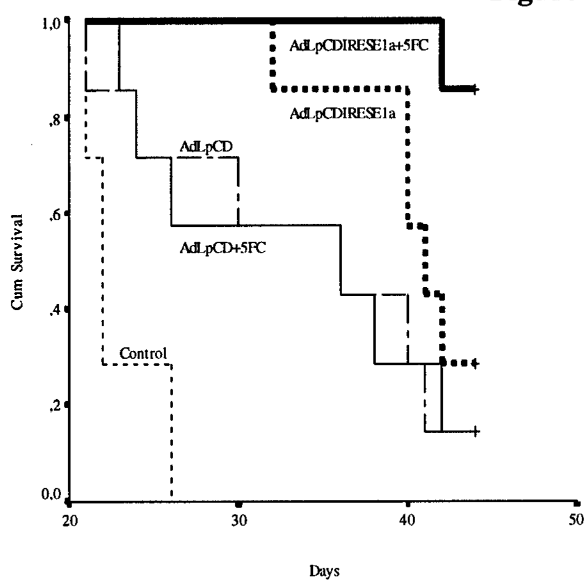


Figure 7



V. Studies of the AdLpCDIRESE1A Vector and Chemotherapy.

V.A. Specificity of expression of the L-plastin promoter driven vector transcription units.

In order to test if the L-plastin driven bicistronic CDIRESE1A transcription unit was expressed in a tumor specific manner in the AdLpCDIRESE1A vector infected cells, we injected either the AdLpCDIRESE1A or the AdCMVCDIRESE1A vectors into normal liver or subcutaneous tumor nodules and stained histological sections of the injected tissue for E1A expression The tumor nodules were positive for E1A whether injected by the AdLpCDIRESE1A or AdCMVCDIRESE1A vectors (Fig. 8c1 and Fig. 8c3) whereas the normal liver tissue was positive for E1A only following injection with the AdCMVCDIRESE1A (Fig. 8c2 and Fig. 8c4). These results show that the expression of the transgenes in the AdLpCDIRESE1A vector infected cells is tumor specific.

V.B. Effect of the AdLpCDIRESE1A vector on IC50 values of CPT-11 SN-38, and 5FU.

We tested if the IC50 of 5FU, CPT-11 and SN-35 was decreased by exposure of the test cells to the AdLpCDIRESE1A vector. When the AdLpCD vector plus 5FC and FA were added to the test cells at MOI 10, the IC50 value of CPT-11 decreased 65-2200 times (Table 1). When the AdLpCDIRESE1A replication competent vector plus 5FC and FA was added to the cells, the decrease in the CPT-11 IC50 was 5-20 times the decrease seen with the AdLpCD replication deficient vector. In contrast, when the AdLpCDIRESE1A vector plus FA without 5FC was combined with CPT-11, the decrease of the IC50 value of CPT-11 was between 7 and 200 fold (data not shown). These studies show that the in vitro activity of CPT-11 and SN-38 is potentiated by the addition of the AdLpCDIRESE1A vector. Moreover, this vector sensitization does not depend on the replication competency of the vector but on the presence of the CD protein and 5FC.

V.C. Replacement of 5FU in the conventional Saltz combination chemotherapy by intratumoral injection of the AdLpCDIRESE1A vector and in vivo 5FC ("Genetic Saltz" Therapy) increases the tumor response and decreases toxicity as compared to the conventional Saltz combination chemotherapy (Animal Model #1).

<u>V.C.1. Response Studies</u>. The growth of the HTB-38 colon cancer cell line in nude mice was suppressed more by intratumoral injection of the AdLpCDIRESE1A vector given in concert with ip 5FC, iv FA and iv CPT-11 chemotherapy (Group 1) than it was by conventional Saltz combination chemotherapy (Group 6) as shown in Fig. 9a. The duration of tumor response among the animals treated with the Genetic Saltz Therapy (AdLpCDIRESE1A/5FC/FA/CPT-11-Group 1) was statistically significantly longer than the duration of the response among animals treated with regimens not containing the AdLpCDIRESE1A/5FC combination ($p<.0001$).

<u>V.C.2. Survival Studies</u>. The mice treated with AdLpCDIRESE1A+5FC+FA+CPT-11 (the Genetic Saltz Therapy or Group 1) lived much longer than did the mice treated with conventional Saltz combination chemotherapy (Group 6) or the other control groups (Fig. 9b). We then tested for the effect of increasing the frequency of the AdLpCDIRESE1A vector injections from once a week to twice a week. A survival advantage was seen in this latter model but this advantage was lost by 12 weeks (data not shown). This suggested that additional cycles of therapy might be one way to increase the success of the outcome.

<u>V.C.3. Toxicity Studies</u>. Importantly, the mice given the conventional 5FU, FA and CPT-11 combination chemotherapy (Group 6) had statistically significantly more diarrhea and slightly more weight loss than that observed in mice treated with the Genetic Saltz Therapy, which involves the combination of AdLpIRESE1A/5FC/FA/CPT-11, as shown in Table 1.

<u>V.D.Response of the HTB-38 colon cancer cells to in vitro infection with the AdLpCDIRESE1A vector infection and in vivo 5FC/FA/CPT-11 chemotherapy (Animal Model #2).</u>

To test if we could improve the outcome of the Genetic Saltz Therapy, we infected the HTB-38 cells with AdLpCDIRESE1A vector in vitro under conditions that would result in infection of 100% of the HTB-38 cancer cells prior to the injection of the tumor cells into the subcutaneous space of the test animals. Following subcutaneous injection of HTB-38 tumor cells which had been infected in vitro with either the

AdLpCDIRESE1A vector, the AdLpCD vector, or the AdWT virus, we treated the mice with the programs outlined in Fig. 8c.

V.D.1. Response Studies. None of the mice treated with the Genetic Saltz Therapy (Group 1) exhibited regrowth of the tumor cells at the injected sites during the 5 months of follow-up whereas the other treatment groups showed regrowth of tumor after chemotherapy (Fig. 9c). In each of the groups in which the colon cancer cell line HTB-38 was infected in vitro with a replication competent vector (Groups 1 and 4) and in which in vivo 5FC (in the case of the AdLpCDIRESE1A) or 5FU (in the case of AdWT) was given, there was a statistically significant reduction in the tumor growth rates (Fig. 9c).

V.D.2. Survival Studies. There was a survival advantage of the Genetic Saltz Therapy (Group 1) as compared to the use of in vivo administration of the conventional Saltz combination chemotherapy (Group 5) as shown in Fig. 9d. When 5FC was deleted from the Genetic Saltz Therapy, the survival advantage of the Genetic Saltz Therapy over the conventional Saltz combination chemotherapy was lost (eg Group 3 in Fig.9d). These results indicated that the outcome of therapy depended on the conversion of 5FC to 5FU within the tumor cells.

Figure 8. Tumor specific adenoviral vectors.

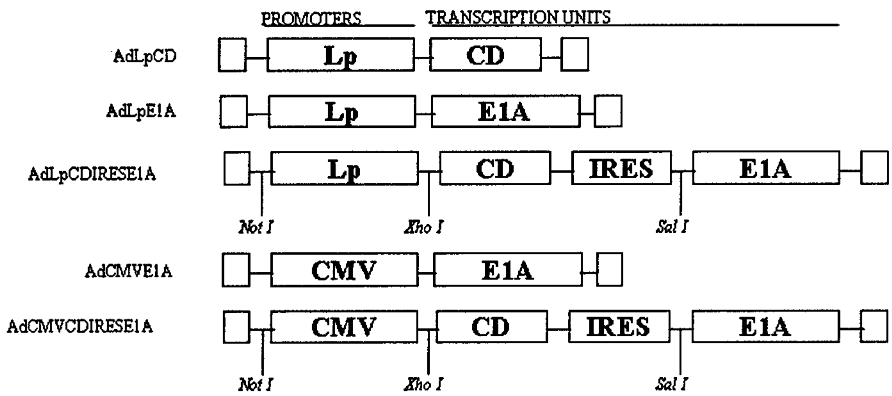


Figure 8a. The maps of the therapeutic transcription units in adenoviral vectors. CD=cytosine deaminase gene; IRES=intraribosomal entry site; E1A= adenoviral replication gene; CMV=cytomegalovirus; Lp=L-plastin.

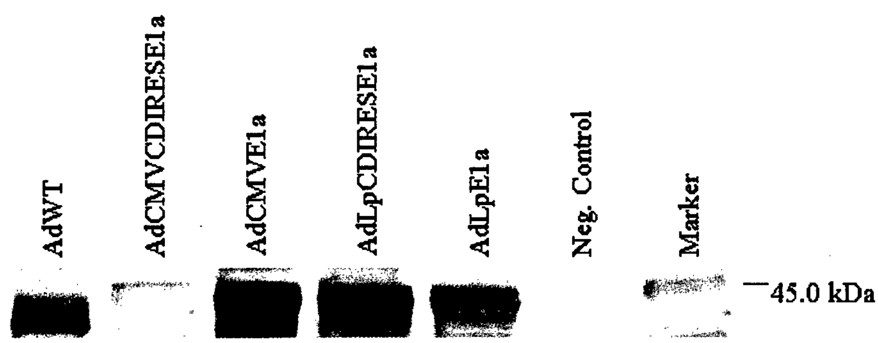


Figure 8b. Western blotting of E1A polypeptides produced in vector infected HTB-38 cells. The E1A region encodes a series of related proteins (35–46kDa).

Figure 8c. Tumor specific expression of the E1A gene following injection with vectors carrying the CMV or L-plastin promoters. In this study, green color indicates expression of E1A peptides

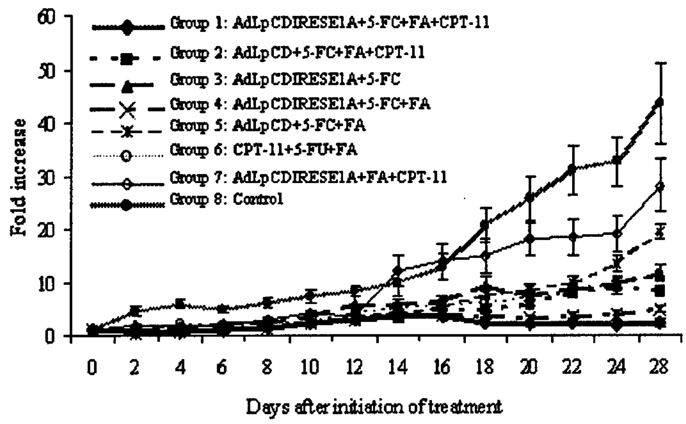
Panel 8c1. In the normal liver injected with the AdLpCDIRESE1A vector, there is no green fluorescence of E1A polypeptides.

Panel 8c2. In the tumor nodule injected with the AdLpCDIRESE1A vector, the green fluorescence is detectable showing that the E1A polypeptides (which are stained green) are produced by the L-plastin driven E1A gene and are present with the nuclei of the tumor cells which are stained red.

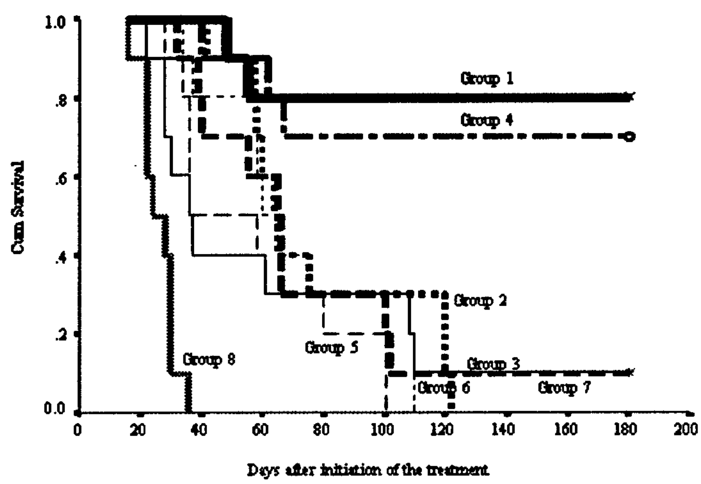
Panel 8c3. The liver was injected with the AdCMVCDIRESE1A vector. The nuclei of the liver cells are stained blue while the E1A polypeptides are stained green in the cells injected with the vector carrying the tumor non-selective CMV promoter. These data show that the normal cells can be infected by the vector and can express E1A peptides in the presence of the CMV promoter.

Panel 8c4. The tumor nodule which was injected with the AdLpCDIRESE1A vector also showed expression of the E1A polypeptides.

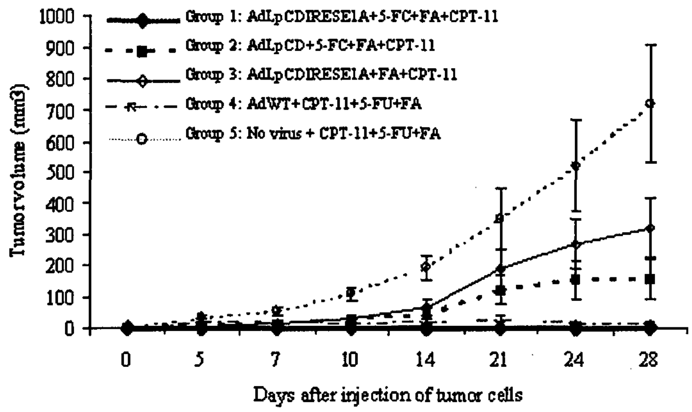
Figure 9. Tumor Volumes and Survival Curves of the Assigned Treatment Groups in Animal Models #1 and #2.



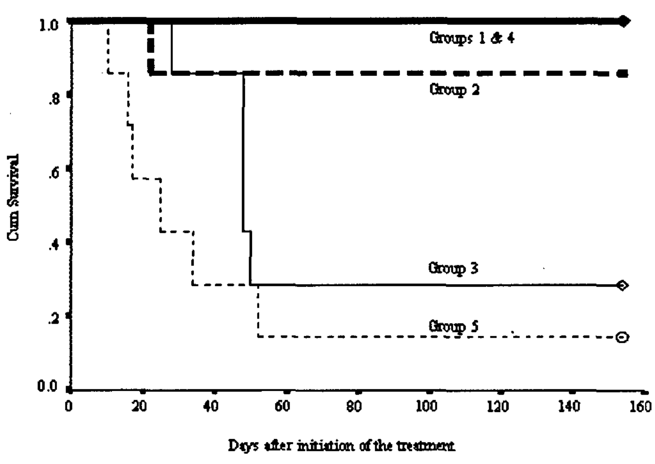
Panel A of Figure 9. Tumor Response of Colon Cancer in Mice Following Treatment in Animal Model #1. The effect of the intratumoral injection of the AdLpCDIRESE1A vector along with intraperitoneal 5-FC daily for 10 days and one day of intravenous FA and CPT-11 ("Genetic Saltz" Therapy-Group 1) on the growth of HTB-38 cells is greater than that of the conventional Saltz combination chemotherapy (Group 6) and of other treatment groups (p<.05).



Panel B of Figure 9. Survival of Mice Following Treatment in Animal Model #1.The effect of the intratumoral injection of the AdLpCDIRESE1A vector along with 10 days of intraperitoneal 5-FC and one day of intravenous FA and CPT-11 ("Genetic Saltz" Therapy-Group 1) on prolongation of survival of mice carrying subcutaneous nodules of HTB-38 cells is greater than that of the conventional Saltz combination chemotherapy-Group 6 (p<.01).



Panel C of Figure 9. Tumor Response of Colon Cancer in Mice Following Treatment in Animal Model #2. The effect of in vitro infection of HTB-38 cells with the AdLpCDIRESE1A vector along with 10 days of intraperitoneal 5FC and one day of intravenous FA and CPT-11 ("Genetic Saltz" Therapy-Group 1) on tumor growth is greater than that of the conventional Saltz combination chemotherapy-Group 5 (p<.05).



Panel D of Figure 9. Survival of Mice Following Treatment in Animal Model #2. The effect of in vitro infection of HTB-38 cells with the AdLpCDIRESE1A vector along with 10 days of intraperitoneal 5-FC and one day of intravenous FA and CPT-11 ("Genetic Saltz" Therapy-Group 1) on prolongation of survival is greater than that of the conventional Saltz combination chemotherapy-Group 5 (p=.0001).

Table 1. The IC50 values of CPT-11 and SN-38 in tumor cells following exposure to the AdLpCD or AdLpCDIRESE1A vectors at 10 MOI.

Tumor cells	$CPT\text{-}11_{IC50}$ (uM)		$SN\text{-}38_{IC50}$ (uM)	
	AdLpCD	AdLpCD-IRESE1A	AdLpCD	AdLpCD-IRESE1A
Ln-CaP	$3.5\pm1.4 \times 10^{-3}$	$2.5\pm0.9 \times 10^{-4}$	$6.8\pm5.14 \times 10^{-12}$	$3.5\pm1.2 \times 10^{-12}$
MCF-7	$6.2\pm2.3 \times 10^{-3}$	$1.4\pm1.5 \times 10^{-3}$	$2.8\pm1.8 \times 10^{-8}$	$3.9\pm1.6 \times 10^{-10}$
Ovcar-5	$2.8\pm1.1 \times 10^{-2}$	$8.2\pm4.4 \times 10^{-3}$	$6.1\pm3.5 \times 10^{-8}$	$5.1\pm1.8 \times 10^{-8}$
HTB-38	$8.8\pm1.7 \times 10^{-3}$	$4.3\pm2.3 \times 10^{-3}$	$7.8\pm4.2 \times 10^{-12}$	$8.2\pm1.3 \times 10^{-12}$

<u>Table 2. Common toxicities seen in the treatment groups of Animal Model #1 (Percent of the total cycles in the first four weeks of treatment).</u>

Groups	Treatment	Diarrhea	Weight**
Group #1	AdLpCDIRESE1A+5FC+FA+CPT-11	2.5	10.4±1.0
Group #2	AdLpCD+5FC+FA+CPT-11	0	3.0±0.6
Group #3	AdLpCDIRESE1A+5FC	0	8.5±0.6
Group #4	AdLpCDIRESE1A+5FC+FA	0	4.1±0.5
Group #5	AdLpCD+5FC+FA	0	6.7±0.6
Group #6	CPT-11+5FU+FA	32.5*	3.0±0.6
Group #7	AdLpCDIRESE1A+FA+CPT-11	2.5	4.8±0.7
Group #8	Control	0	8.6±0.6

- *The mice in the Saltz regimen (CPT-11+5-FU+FA) which is Group 6 had significantly more episodes of diarrhea (grade 1 or more) than the other groups (p=.001)
- **Denotes the percent change of weight during the first cycle of the treatment.

Key Research Accomplishments:

A. Development of a new concept in chemotherapy of colon cancer which involves the replacement of one drug (5FU) in the Saltz conventional combination chemotherapy 3 drug regimen (5FU, CPT-11 and folinic acid (FA), with the Ad-LpCDIRESE1A vector and 5FC (Genetic Saltz Chemotherapy) plus CPT-11 and FA.

B. Study of this regimen in an animal model of colon cancer showing that the newly developed Genetic Saltz Chemotherapy produces longer remission duration, longer survival and less toxicity than the conventional Saltz combination chemotherapy.

Reportable Outcomes - Papers Completed During the Entire Funding Period of the Grant:

1. Chung I, Crystal RG and **Deisseroth AB**. Adenovirus specific for neoplastic cells. Cancer Gene Therapy, 6: 99-106, 1999.
2. Peng XY, RutherfordT, Won JH, PizzornoG, ZeltermanD, Sapi E, Kaczinski B, Leavitt J, Crystal R, and **Deisseroth A.** L-plastin promoter for adenoviral tumor-specific gene expression in ovarian ca. Cancer Research 61:4405-4413, 2001.
3. Zhang, L, Akbulut, H, Tang, Y, Peng XY, Pizzorno G, Sapi, E, Manegold, S and **Deisseroth, A**. Adenoviral vectors with E1A regulated by tumor specific promoters are cytolytic for breast cancer. Molecular Therapy 6:386-393, 2002.
4. Akbulut, H, Zhang, L, Tang, Y, and **Deisseroth, A**. The cytotoxic effect of adenoviral vectors carrying L-plastin promoter regulated E1A and CD genes in cancers of the breast and colon. Cancer Gene Therapy, 10: 388-395, 2003.
5. Akbulut, H, and **Deisseroth, A**. Prevention of Cervical Cancer. In Progress in Oncology 2002. Eds. VT DeVita,Jr, S. Hellman, and SA Rosenberg. Jones and Bartlett Publishers, Boston, MA pp. 251-269, 2002.
6. Zhang, L, Tang, Y, Linton PJ, and **Deisseroth, A.** Injection of Ad vector encoding secretable form of TAA/CD40L fusion protein induces T cell dependent immune response for against tumor cells. Accepted for Communication, PNAS, 2003.
7. Akbulut, H, Tang, Y, Maynard, J, and **Deisseroth, A.** Reduced toxicity and increased efficacy of Saltz regimen which utilizes vector mediated tumor specific delivery of 5FU. Submitted 2003.
8. **Deisseroth A**, Tang YC, Akbulut H, and Liu YZ. Vector Targeting of Cancer. In: Critical Reviews in Oncology/Hematology, MS Aapro, Ed., invited chapter, in prep., Elsevier House, Shannon, Co. Clare, Ireland, 2003.

Conclusions:

The conclusions that can be reached from this research study are as follows:

1. The L-plastin promoter is a strong tumor specific transcriptional promoter even when placed in the backbone of the adenoviral vector.
2. The replication incompetent Ad-LP-CD vector can sensitize tumor cells to the effects of chemotherapy.
3. The Ad-Lp-E1A produces a tumor specific cytolytic effect on tumor cells.
4. The AdLpCDIRESE1A vector displays greater toxicity to tumor cells that either of the single transcription unit vectors.
5. When the Saltz Combination Chemotherapy Regimen of 5FU, CPT-11 and folinic acid (FA) is modified so that 5FU is replaced by the AdLPCDIRESE1A vector and 5FC and given along with CPT-11 and FA, the survival of tumor bearing mice is prolonged, the response duration of subcutaneous nodules of tumor is prolonged and the gastrointestinal toxicity of the regimen, as measured by diarrhea, is decreased in tumor bearing mice.
6. If the fiber protein of this vector can be modified such that the binding of the AdCDIRESE1A is specific to tumor cells or tumor vasculature, then this vector could be given intravascularly for the treatment of metastatic cancer.

Appendices:

1. Chung I, Crystal RG and **Deisseroth AB**. Adenovirus specific for neoplastic cells. Cancer Gene Therapy, 6: 99-106, 1999.
2. Peng XY, RutherfordT, Won JH, PizzornoG, ZeltermanD, Sapi E, Kaczinski B, Leavitt J, Crystal R, and **Deisseroth A.** L-plastin promoter for adenoviral tumor-specific gene expression in ovarian ca. Cancer Research 61:4405-4413, 2001.
3. Zhang, L, Akbulut, H, Tang, Y, Peng XY, Pizzorno G, Sapi, E, Manegold, S and **Deisseroth, A**. Adenoviral vectors with E1A regulated by tumor specific promoters are cytolytic for breast cancer. Molecular Therapy 6:386-393, 2002.
4. Akbulut, H, Zhang, L, Tang, Y, and **Deisseroth, A**. The cytotoxic effect of adenoviral vectors carrying L-plastin promoter regulated E1A and CD genes in cancers of the breast and colon. Cancer Gene Therapy, 10: 388-395, 2003.
5. Akbulut, H, Tang, Y, Maynard, J, and **Deisseroth, A.**. Reduced toxicity and increased efficacy of Saltz regimen which utilizes vector mediated tumor specific delivery of 5FU. Submitted 2003.

Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cells

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The objective of this study was to develop an adenoviral vector system that would generate a pattern of expression of exogenous therapeutic genes appropriate for the treatment of ovarian cancer. For this purpose, we have generated a replication-deficient recombinant adenoviral vector, AdLPLacZ, which contains the human L-plastin (LP) promoter (LP-P) driving the *Escherichia coli LacZ* gene. LP is constitutively expressed at high levels in malignant epithelial cells but is not expressed in normal tissues, except at low levels in mature hematopoietic cells. Because adenoviral vectors infect early hematopoietic multilineage precursor cells only poorly or not at all, this vector would be of use in the peritoneal cavity and *in vitro* for marrow purging. We first analyzed the expression of the *LacZ* reporter gene in ovarian and breast cancer cell lines, normal fibroblasts, and leukemia cell lines using the adenoviral vector in which the *LacZ* gene is governed by the LP-P promoter (AdLPLacZ) or in which the *LacZ* gene is governed by the cytomegalovirus (CMV) promoter (AdCMVLacZ). We found equivalent and high levels of expression of β-galactosidase (β-gal) by AdLPLacZ and AdCMVLacZ vectors in the breast or ovarian cancer cell lines as well as in a fibrosarcoma cell line, indicating that the adenoviral vectors infected these cells and expressed their transgenes equally with the LP and CMV promoters. Expression of the *LacZ* gene with the CMV vector but not with the LP-P vector was observed in experiments with normal fibroblasts, indicating that the vectors infected the cells, but that the LP-P was not active within them. In hematopoietic cells such as U937 cells, no measurable β-gal activity was detected in cells infected either by AdLPLacZ or by AdCMVLacZ, indicating that the adenoviral vectors were not infecting the cells. Although β-gal activity was observed in fresh ascitic ovarian cancer cells after infection with adenoviral vectors containing CMV or the LP promoters, β-gal activity was detected in a portion of a biopsy of normal peritoneum when the tissues were exposed to the AdCMVLacZ vector, but not when tissues were exposed to the AdLPLacZ vector. These results suggest that the transcription of therapeutic genes in cells infected by the AdLP vectors would be restricted to LP expression-positive ovarian carcinoma cells but would not be seen in the normal mesothelial cells of the peritoneal cavity. This possibility implies that adenoviral vectors carrying therapeutic genes driven by the LP-P would be of use for the intracavitary treatment ovarian cancer.

***Key words:** Ovarian cancer; LacZ; L-plastin; tumor specific.*

Gene therapy represents a potentially novel approach to cancer treatment, in which the transfer of genetic material into a specific cell type alters the phenotype of the target cells[1-3] in a way that improves the outcome of therapy. Adenoviral vectors have become the most widely used vector for the delivery of prodrug activation transcription units in the field of cancer gene therapy.[4-8] However, one of the limitations of this vector system for cancer gene therapy may be its broad cellular host range, which results in toxicity to both the tumor cells and the surrounding normal cells. One way to circumvent this limitation would be through the use of a tumor- or tissue-specific promoter for the therapeutic gene carried by the vector, which is active in the target tumor cells but not in the normal cells. Comparative examination studies of protein synthesis in normal and neoplastic human fibroblasts that were conducted in the laboratory of J.L.[9-12] led to the discovery of *L-plastin (LP)*, a gene that codes for an actin-binding protein, which is expressed at high levels in human epithelial cancer cells.

In their review of solid tumors, LP was found to be a marker expressed at high levels in the majority of human

Received January 12, 1998; accepted May 13, 1998.

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cancer cells of nonhematopoietic origin.[9-12] A survey of simian virus 40-transformed human fibroblasts and human sarcoma cell lines as well as human carcinoma cell lines has demonstrated that the *LP* gene was transcriptionally active, although at widely varying degrees, in nearly all of the human cancer cells tested.[9-12] In particular, high levels of LP synthesis are found in tumors derived from female reproductive organs.[12] In addition, the *LP gene* seems to be transcriptionally regulated through the 5.1 kilobases (kb) of the 5'-regulatory region.[11-12] Because the adenoviral vector has a wide host range in epithelial cells, we proposed using the LP promoter (LP-P) in an adenoviral vector to restrict the expression of the adenoviral vector therapeutic gene to the LP-positive carcinoma cells, so as to avoid expression in normal cells. In the present study, we generated a replication-deficient adenoviral vector carrying the *LacZ* gene in a transcriptional unit governed by a truncated LP-P (a 2.4-kb subset of the 5'-promoter region of the *LP gene*) to accommodate both the *LP-P* and therapeutic genes. We subsequently studied the use of this LP LacZ adenoviral vector in various human cell lines and patient samples to determine the pattern of its transgene expression in neoplastic and normal cells.

MATERIALS AND METHODS

Generation of AdLPLacZ

We obtained a replication-incompetent adenoviral type 5-based vector from which the E1 and E3 genes were deleted (provided by the laboratory of R.G.C., The New York Hospital, Cornell Medical Center).[3-5] The AdLPLacZ vector is a modification of the vector obtained from the this laboratory, in which the human LP-P and the *Escherichia coli* LacZ gene that codes for β-galactosidase (β-gal) have been introduced by the Deisseroth laboratory into the E1 region. The first step in the generation of AdLPLacZ was to construct an E1 shuttle vector, pLPLacZ, which contains the expression cassette of the *LacZ* gene under the control of the human LP-P. This was accomplished as follows: we first isolated a *ScaI* 2.4-kb fragment (i.e., −2265 to +118 base pairs (bp)) of the human LP-P from the pHLPr-β-gal-Neo plasmid, which was obtained from J.L. (Palo Alto Medical Research Institute),[9-12] as shown in Figure 1. At the beginning of the construction, the vector was identical with that outlined by Hirschowitz et al.[5] We then ligated the *ScaI* 2.4-kb fragment to the *HincII* site of pBluescript II SK$^-$ (Stratagene, La Jolla, Calif), to produce pLPSK (see Fig 1). The *SpeI/XhoI*-digested 2.4-kb fragment of pLPSK was then ligated to the *SpeI/XhoI*-digested pCMVβ-P (obtained from R.G.C.)[3-5] after removing the cytomegalovirus (CMV) enhancer/promoter to obtain pLPLacZ (see Fig 1). The adenovirus vector AdLPLacZ was produced by homologous recombination using standard techniques.[3] For homologous recombination to occur, pBHG10 (Microbix Biosystems, Ontario, Canada) and pLPLacZ were cotransfected into 293 cells by the calcium phosphate coprecipitation method. Individual plaques were screened, and the identity of each as AdLPLacZ was verified (see Fig 2) by amplifying part of the sequences of the LacZ gene and Ad5 by polymerase chain reaction (PCR). The sequences of the primers used for the screen were as follows: the forward primer of Ad5, 5'-TCGTTTCTCAGCAGCTGTTG-3', and the reverse primer of Ad5, 5'-CATCTGAACTCAAAGCGTGG-3', were located at 11 and 13.4 map units of the Ad5 genome, respectively, which generated a 860-bp PCR product as published previously.[13] The primer set for the *LacZ* gene was designed according to the sequence information for the pCMVβ-gal plasmid provided by Clontech (Palo Alto, Calif). The forward primer of *LacZ* gene included from −2072 to −2092 bp (5'-CCTGCTGATGAAGCAGAAC-3'); the reverse primer was from −2340 to −2358 bp (5'-CGATTAGTGCTGCGCGACA-3'), which generated a 287-bp PCR product. The results of the PCR assays (see Fig 2) documented the introduction of the *LacZ* transcription unit into the adenoviral vector.

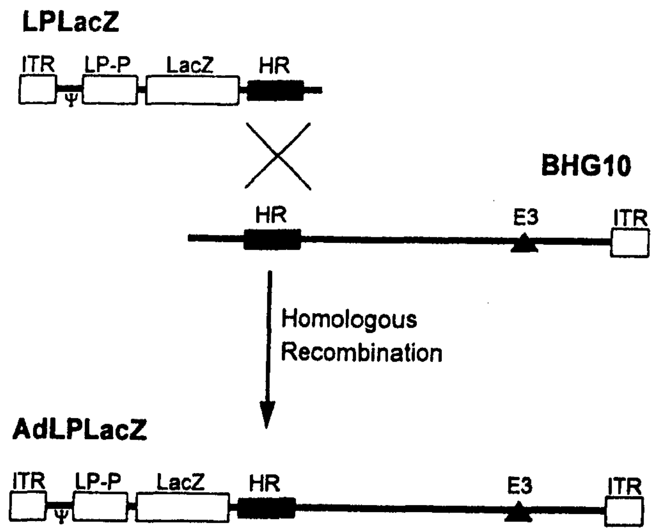

Figure 1. Assembly of AdLPLacZ adenoviral vector by the homologous recombination method. LPLacZ is a shuttle vector that contains an adenoviral inverted terminal repeat (ITR) and packaging signal (Ψ), the *LacZ* gene driven by the LP-P, and some adenoviral vector sequences that are homologous in sequence (HR) to those present in the BHG10 adenoviral vector plasmid clone. These HR sequences, when combined with the pBG10 adenoviral vector plasmid, produce a complete replication-incompetent adenoviral vector (AdLPLacZ), which contains the *LacZ* gene driven by the LP-P. The BHG10 plasmid was obtained from the laboratory of Frank Graham. (Microbix Biosystems, Ontario, Canada) It contains all of the adenoviral genes 3' to the E1 gene, except for the *E3* gene, which has been deleted. There is another ITR at the very 3' end of the vector. The complete recombinant vector is designated AdLPLacZ.

AdLPLacZ and control virus (AdCMVLacZ), which contained a CMV promoter driving the *LacZ* gene (obtained from the laboratory of R.G.C.), were propagated in 293 cells and recovered 36 hours after infection by five cycles of freezing/thawing of the infected cells. All viral preparations were purified by CsCl density centrifugation, dialyzed, and stored in dialysis buffer (10 mM tris(hydroxymethyl)aminomethane-HCl (pH 7.4) and 1 mM $MgCl_2$) at −70°C before use. Titers of the viral stocks were determined by plaque assay using 293 cells by standard methods.[14]

Cell culture

Cells were maintained in Iscove's modified essential medium (Biofluids, Rockville, Md) supplemented with 10% fetal bovine sera (FBS) and 2 mM glutamine (in the case of 293 cell line); in Dulbecco's modified Eagles' medium/F12 (Life Tech-

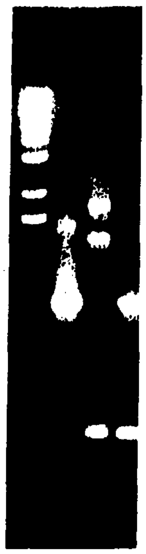

Figure 2. PCR analysis of recombinant adenoviral vector with LP-P/*LacZ* gene. DNA was isolated from plaques resulting from calcium phosphate transfection of the AdLPLacZ shuttle vector and the pBHG10 adenoviral vector with a deletion in the E1 gene region. The homologous recombination event would take place such that the LP/*LacZ* gene would be inserted into the adenoviral vector in the *E1* gene region. PCR was performed on this DNA as outlined in *Materials and Methods*. The adenoviral vector strain 5 primers generate an 860-bp fragment, and the LacZ primers generate a 287-bp fragment. Lane 1, molecular weight markers; lane 2, control for the adenoviral vector; lane 3, control for the LacZ shuttle vector; lane 4, recombinant vector. We conclude that the desired vector was obtained.

nologies, Gaithersburg, Md) supplemented with 10% FBS (in the case of the HT-1080, WI-26-VA4, CCD-944SK, CCD-862SK, PA-1, BT-20, MDA-MB-468, SKOV-3, and IGROV-1 cell lines); in Dulbecco's modified Eagle's medium/F12 supplemented with 10% FBS and 10 μg/mL of insulin (in the case of the MCF-7, MDA-MB-436, and OVCAR-3 cell lines), and in RPMI 1640 (Life Technologies) supplemented with 10% FBS (in the case of the HL-60 and U937 cell lines). All cell lines were purchased from the American Type Culture Collection (Manassas, Va).

Preparation of patient samples

Samples of ovarian cancer or normal peritoneal surface tissue were obtained by P.E.S. as incidental specimens during diagnostic and therapeutically indicated surgical procedures for the treatment of ovarian cancer. The procurement of these samples was performed as part of protocols approved by the Yale Human Investigations Committee. The tissue samples were minced with scissors, treated with disaggregation medium and collagenase, and rinsed; viable cell counts were determined by trypan blue exclusion. Cells were then immediately exposed to the vector for a 24-hour period and subsequently stained for LacZ gene activity.

Infection of cells and detection of β-gal activity

For infection and the detection of β-gal activity by staining, cells were exposed to complete tissue culture medium supplemented at 10% by volume with AdLPLacZ crude viral lysate of an overnight culture. After 24 hours, cells were fixed at room temperature for 10 minutes with a solution of 2% formaldehyde and 0.2% glutaraldehyde, washed three times with phosphate-buffered saline, and exposed overnight to 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (1 mg/mL) in phosphate-buffered saline containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgSO_4$ at 37°C.[15] For flow cytometric analysis and sorting (FACS) of cells for β-gal activity, cells (1×10^5 cells/well in a 24-well plate) were infected with AdLPLacZ or AdCMVLacZ at a multiplicity of infection of 25 or 50 in medium containing 2% FBS for 90 minutes. At 24 or 48 hours after the initial infection, cells were subjected to FACS-gal analysis as described by Roederer et al.[15] Briefly, after harvesting by centrifugation (HL-60 and U937) or by trypsinization, cells were resuspended with 50 μL of staining medium (RPMI 1640 supplemented with 4% FBS and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4) containing chloroquine and incubated for 20 minutes at 37°C. Cells were then exposed to 50 μL of 2 mM of fluorescein di-β-D-galactopyranoside for 60 seconds, following which 1 mL of ice-cold Quench solution (staining solution with propidium iodide) was added; subsequently, cells were further incubated on ice for 1 hour. After adding phenylethyl β-D-thiogalactopyranoside, cells were subjected to FACS analysis.

RESULTS

Generation of AdLPLacZ

We have generated a recombinant adenoviral vector, AdLPLacZ, in which the *E. coli* LacZ reporter gene is placed downstream of a 2.4-kb DNA sequence (−2263 to +118 bp with respect to the transcription start site) that was taken from the human LP 5' transcriptional regulatory region (Fig 1). The E3 region and most of the E1 region were removed from this vector.[4] The expression cassette containing the LP-P and the LacZ gene replaces the E1 region of the adenovirus strain 5. Lin et al[11] have indicated that activation of the LP gene in tumorigenesis is governed by *cis*-acting elements present in 5.1 kb of DNA located in the transcriptional regulatory region immediately 5' to the LP gene. However, we incorporated a 2.4-kb DNA sequence of the LP gene (−2263/+118) in this study, largely attributable to the limited capacity of the adenoviral vector, pBHG10, in which a maximum of 7.5 kb of exogenous sequence can be inserted. PCR analysis of DNA isolated from plaque-purified clones of this vector (see Fig 2) documented the successful introduction of β-gal into these vectors. We have tested this AdLPLacZ vector in 293 cells by a staining method to determine whether this vector could produce functional β-gal enzyme. Intense blue staining was observed in 293 cells that have been infected with the AdLPLacZ crude viral lysate (data not shown).

Testing of AdLPLacZ in normal and neoplastic cells

To test the ability of the 2.4 kb of LP-P to direct the expression of the *LacZ* transgene in a tumor cell-specific manner, we infected various human cell lines with the AdLPLacZ crude viral lysate and monitored β-gal activ-

Table 1. Comparison of X-Gal Staining in Various Human Cell Lines After Infection with Ad.LP.LacZ

Cell lines	X-Gal staining
Ovarian carcinoma	
PA-1	+
OVCAR-3	+
SKOV-3	+
Hematopoietic cells	
U937	−
HL-60	−
Fibrosarcoma	
HT-1080 Simian virus-transformed	+
WI-26-VA4 Human skin fibroblast	−
CCD-944SK	−
CCD-862SK	−
Breast carcinoma	
MCF-7	+
BT-20	+
MDA-MB-436	+
MDA-MB-468	+

Table 2. FACS-Gal Analysis

Cell lines	FACS-gal analysis LP/CMV (%)	Range (LP, CMV)
Ovarian carcinoma		
OVCAR-3	0.68	(30–95, 50–95)
SKOV-3	1.45	(20–70, 10–45)
Hematopoietic cells		
U937	0.8	(1–5, 1–5)
Fibrosarcoma		
HT-1080	0.7	(20–90, 40–80)
Simian virus-transformed		
WI-26-VA-4	0.27	(5–20, 40–50)
Human skin fibroblast		
CCD-944SK	0.07	(1–2, 15–45)
CCD-862SK	0.11	(1–2, 15–30)
Breast carcinoma		
BT-20	0.23	(10, 30–50)
MDA-MB-468	0.95	(75–97, 80–97)

Data were obtained from six independent experiments. The percentage of cells expressing β-gal, determined by FACS-gal analysis, for the LP and CML vectors, is indicated on the right side of the Table (LP, CMV). The relative activity, as measured by the ratio of the percentage of cells expressing β-gal with the LP and CMV promoters, is on the left side of the Table (LP/CMV (%)).

ity by a chrometric staining with X-Gal as described in *Materials and Methods*. The results of this experiment are shown in Table 1. Intensely staining blue color reactions catalyzed by the LP-*LacZ* adenoviral vector were produced in all of the human ovarian carcinoma cell lines tested, such as PA-1, OVCAR-3, and SKOV-3, as well as in human mammary carcinoma cell lines such as MCF-7, BT-20, MDA-MB-436, and MDA-MB-468. However, no such staining was detected in normal human skin fibroblast cell lines such as CCD-862SK and CCD-944SK, even at 3 days after initiation of the staining reaction (see Table 1). A human fibrosarcoma cell line, HT-1080, which expresses a high level of endogenous *LP*,[12] developed a dark-blue staining upon infection with this viral vector, whereas the cell line WI-26-VA4, which is not known to express *LP* from its endogenous gene,[12] exhibited no staining by this method. Because *LP* is constitutively expressed in normal mature hemopoietic cell lines, we assumed that the human leukemia cell lines U937 and HL-60 might express abundant LP. However, only a small percentage of cells exhibited a blue color upon exposure to AdLPLacZ (see Fig 3 and Tables 1 and 2). This low level of LacZ reactivity may be attributable to the low infectivity of early hematopoietic cells for the adenoviral vector (see below).

To measure the transduction efficiencies of the AdLP vector and the relative strength of LP-P in cell lines, we performed FACS-gal analysis of the adenoviral vector-infected cells following exposure to the AdLPLacZ or AdCMVLacZ vectors at a multiplicity of infection (ratio of infectious viral particles to nucleated cells) of 50. At 24–48 hours postinfection, cells were subjected to FACS-gal analysis. In Figure 3, we present the representative results of FACS analysis of β-gal staining of the infected cells.

Less than 1% of the CCD-862SK and CCD-944SK normal human skin fibroblast cell lines expressed β-gal upon infection with AdLPLacZ, in which the LP-P was driving the *LacZ* gene; however, ≥25% of the cells are infectable by the adenoviral vector, as suggested by the blue color, which develops when the same cells are infected with the control vector, AdCMVLacZ, in which the CMV promoter is driving the *LacZ* gene. These results indicated that the absence of *LacZ* expression in normal skin fibroblasts infected with the LP-β-gal adenoviral vector did not result from the low infection efficiency of AdLPLacZ in normal fibroblasts, but rather from a low transcriptional activity of the LP-P in fibroblast cell lines.

In contrast, in a cell line known to express high levels of LP (i.e., HT-1080), nearly 90% of the cells were positive for infection by the AdLPLacZ vector and for expression of the *LacZ* gene, as measured by positivity in the β-gal staining assay. Infecting SKOV-3, a human ovarian carcinoma cell line, with the AdLPLacZ and AdCMVLacZ vectors generated 70–100% and 45% positivity of cells in the β-gal assay, respectively. A high percentage of β-gal-expressing cells was observed in MDA-MB-468 cells (a human breast carcinoma cell line) following exposure to either AdLPLacZ or AdCMVLacZ. These results suggest that the LP-P is almost as active as the CMV promoter in ovarian and breast cancer cell lines.

In contrast, only ~5% of the U937 human leukemia cells were positive in the β-gal assay when these cells were exposed to either the AdLPLacZ or the AdCMV-LacZ vector, confirming the fact that adenoviral vector infects early hematopoietic cells very inefficiently or not at all.[16–18] These results, which were obtained from six independent experiments, are summarized in Table 2.

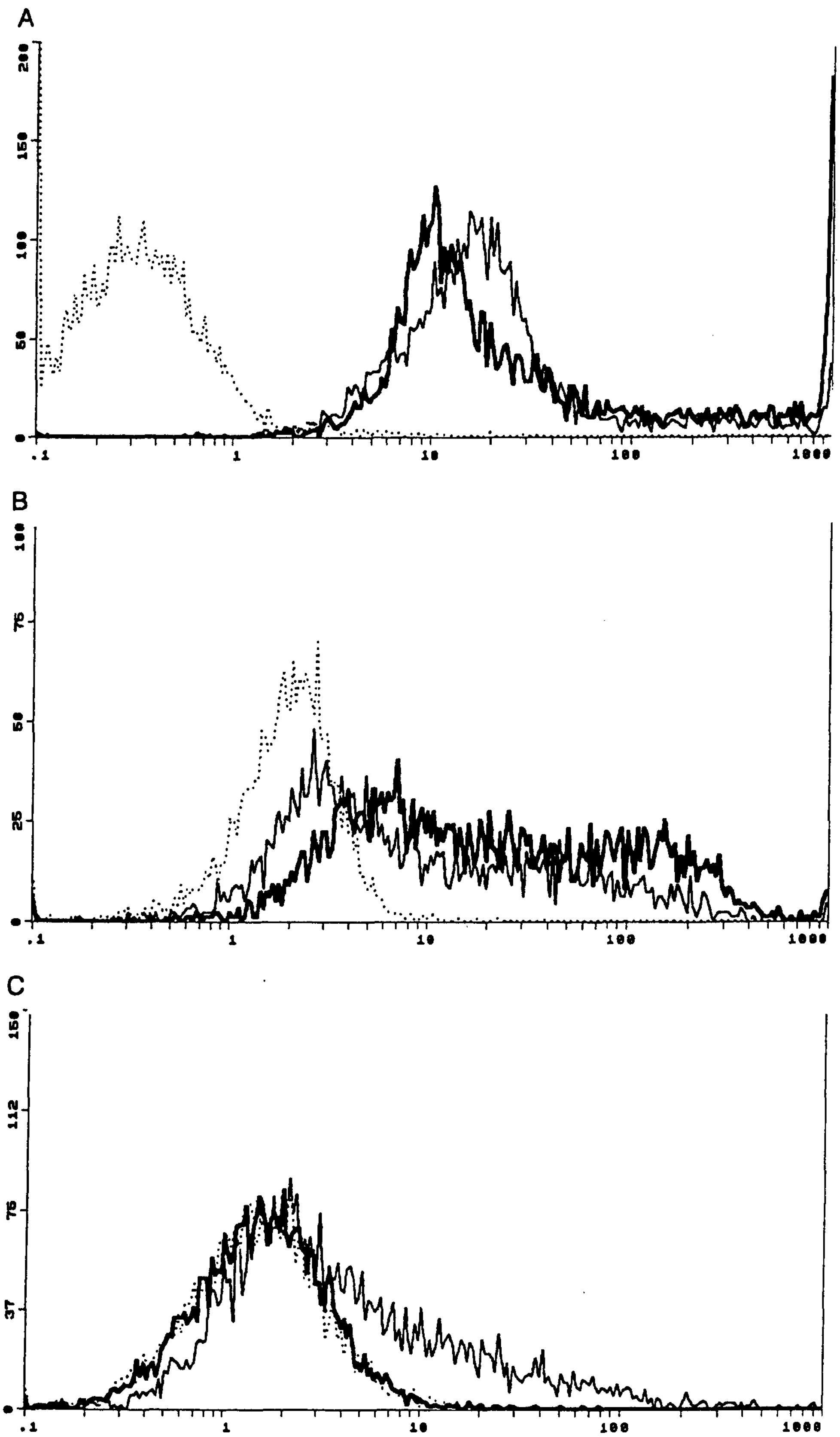

Figure 3. FACS-gal analysis of the cell lines exposed to AdLPLacZ and AdCMVLacZ adenoviral vectors. Cell lines derived from patients with: **A**, breast cancer (MDA-MD-468); **B**, ovarian cancer (SKOV-3); **C** and **D**, skin fibroblasts (CCD-862SK and CCD-944SK); **E**, hematopoietic leukemia cells (U937); and **F**, simian virus 40-transformed cells (HT-1080) were exposed to either the AdL-PLacZ vector (dark solid line) or the AdCMVLacZ vector (light solid line) and compared with cells exposed to no vector (light dotted line) under the conditions analyzed in *Materials and Methods*. The cells were then stained by the X-Gal reaction and analyzed by a fluorescence detection scanner.

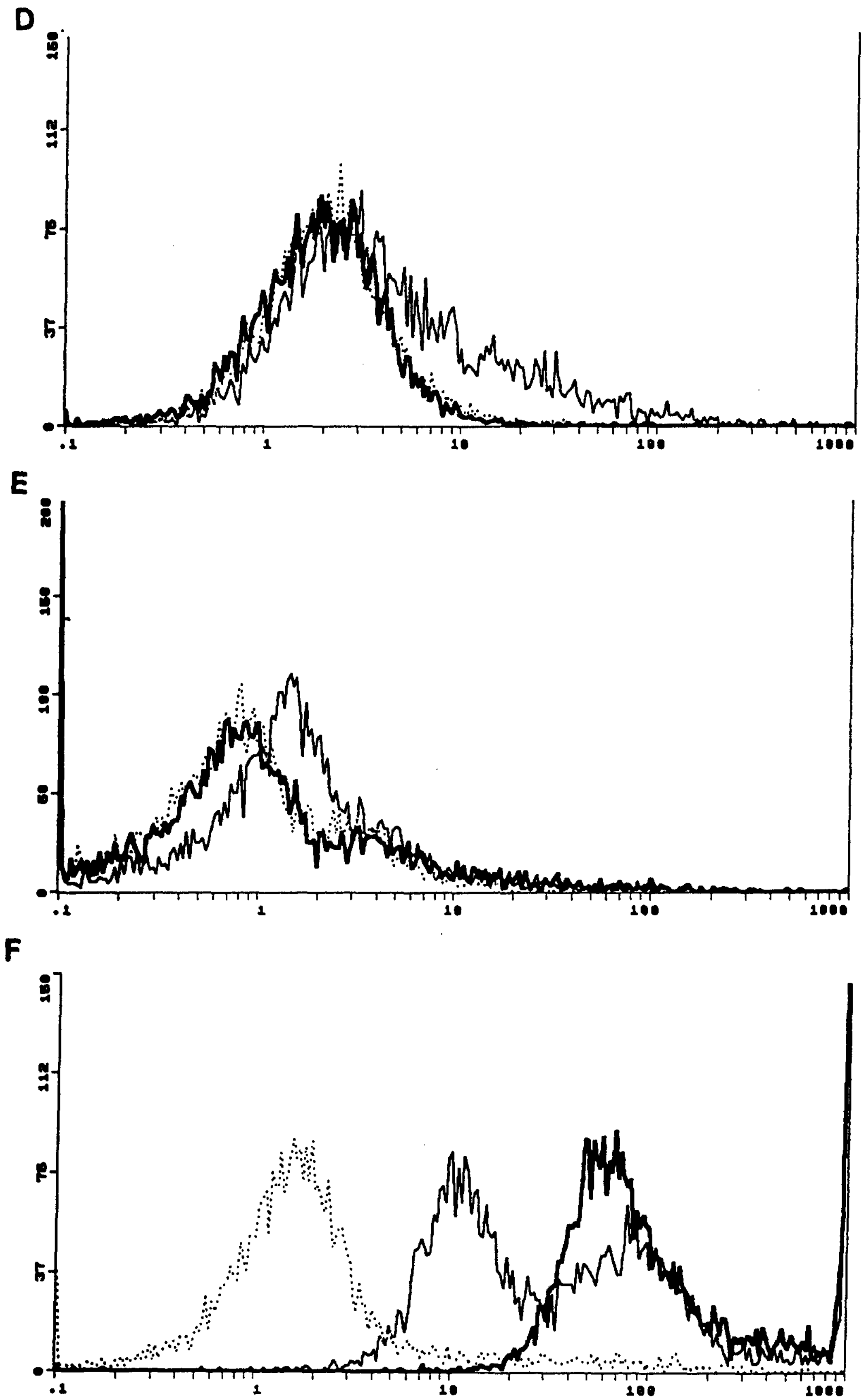

Figure 3. Continued

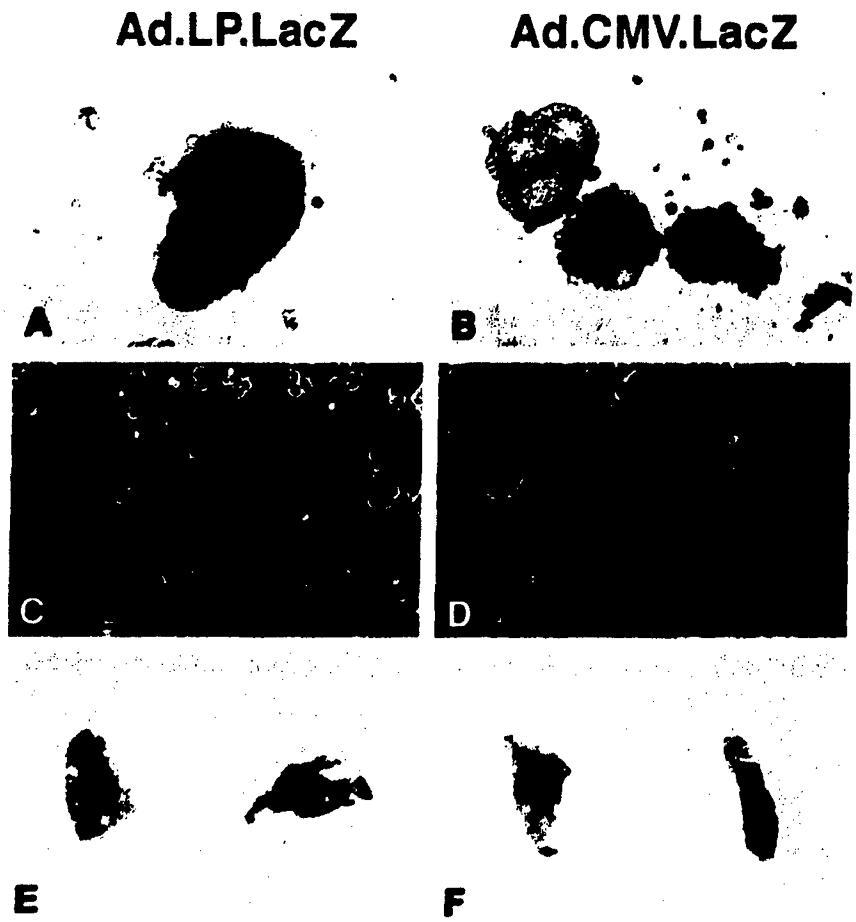

Figure 4. X-Gal staining studies of normal mesothelial cells and malignant ascites from an ovarian cancer patient. Fresh samples from patients were exposed to 0.5 mL of medium containing 150 million plaque-forming units of the adenoviral vector (except for **E** and **F**, in which 50 million plaque-forming units were used) for 90 minutes, washed, and incubated in the absence of the vector for another 24 hours; next, samples were exposed to the conditions of the X-Gal staining. Ascitic ovarian cancer cells were infected with either the AdLPLacZ (**A** and **C**) or the AdCMVLacZ (**B** and **D**) vectors. Biopsy cells from the mesothelium of patients undergoing surgical procedures were exposed to either the AdLPLacZ (**E**) or AdCMVLacZ (**F**) vectors.

The relative activities of the LP and CMV transcriptional promoters in infected cell lines, when measured by the percentage of β-gal-positive cells, are also documented by these data. In summary, the AdLP vector system could direct the expression of a heterologous gene in a neoplastic cell-dependent manner. These data suggest that the level of expression would be dependent upon the endogenous level of LP expression.

Testing of AdLPLacZ in normal peritoneum and ascitic ovarian cancer cells from patients

To determine the susceptibility to infection by adenoviral vectors and the level of expression of transgenes driven by the LP-P in ovarian cancer cells and normal mesothelial peritoneal lining cells obtained from patients, we incubated ascitic ovarian cancer cells and a portion of a biopsy of the normal mesothelial cells of the peritoneum with either the AdLPLacZ or the AdCMVLacZ vectors overnight. At 2 days after infection, β-gal expression was determined by X-Gal staining. As shown in Figure 4, LacZ expression was visible in the ascitic ovarian cancer cells that had been infected with AdLPLacZ (Fig 4, A and C) as well as with AdCMVLacZ (Fig 4, B and D). In the portion of the peritoneal biopsy that contained normal mesothelial cells, however, the cells did not produce any blue staining upon infection with the vector in which the LacZ gene is under the control of the LP-P (Fig 4E); cells that had been infected with a vector in which LacZ gene is driven by the CMV promoter expressed the β-gal in a dose-dependent manner (Fig 4F).

In summary, an adenovirus vector with a CMV promoter infected and expressed the *LacZ* gene in ascitic ovarian cancer cells as well as in normal peritoneal mesothelial cells. However, an adenoviral vector with the LP-P directing the *LacZ* gene was expressed in ovarian cancer cells but not in the normal mesothelial cells obtained from a single patient at the time of a therapeutically indicated surgical procedure. These studies indicated that the ascitic ovarian cancer cells and normal mesothelial cells were both infectable by the adenoviral vectors, because they both expressed the *LacZ* gene driven by the CMV promoter in an adenoviral vector. In contrast, the LP-P-driven *LacZ* adenoviral transcription unit was expressed in the ovarian cancer cells but not in the normal mesothelial cells.

DISCUSSION

Usually the extent of expression of therapeutic transgenes in cells infected by the adenoviral vectors carrying those genes in a transcription unit driven by the CMV promoter is determined largely by infection efficiency. The sensitivity of a cell to infection by the adenoviral vector depends not only upon the number of receptors on the cell surface for the fibrillar protein, which mediates binding, but also upon the presence of the $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins that bind to the adenovirus penton base proteins, thereby mediating the uptake of the adenovirus at the cell membrane level and its release from postuptake endosomes.[19,20] As an example, epithelial cells are readily infectable by the adenoviral vector, but early hematopoietic cells, because they do not contain the requisite integrin receptors, are not readily infectable without an induction of differentiation of these cells, which increases the levels of the necessary integrins on the cell surface (see Refs. 19–21). However, in this study, we have generated an adenoviral vector system that contains 2.4 kb of LP-P regulating the expression of the *LacZ* reporter gene in neoplastic cells. In cells exposed to this vector, the expression of the transgene is governed not only by the infectability of cells by the vector but also by the activity of the LP-P in the infected cells. We have demonstrated *in vitro* that the 2.4 kb of LP-P nearest the transcription initiation site of the gene could direct heterologous gene expression preferentially in neoplastic, but not in normal, mesothelial cells. In addition, we have shown that the LP-P activity has a strength of transcriptional activity in neoplastic epithelial cells that is comparable with that seen with the CMV promoter. Thus, the AdLP vector carrying a prodrug activation chemotherapy sensitization transcription unit may have significant utility in the chemosensitization of the ovarian cancer cells present as implants on the peritoneal surface or as ascites to intraperitoneal chemotherapy, because the LP-P in the studies presented in this paper was very active in ovarian cancer cell lines and in fresh ascitic ovarian cancer cells but was not active in cell lines of mesodermal origin or in normal peritoneal mesothelial cells.

ACKNOWLEDGMENTS

We recognize the support of the Bush Leukemia Research Fund and the Anderson Chair for Cancer Treatment and Research at the M.D. Anderson Cancer Center (to A.B.D.), the Hull Development Fund of the Yale Comprehensive Cancer Center, and the Ensign Professorship of Medicine of the Department of Internal Medicine at the Yale University School of Medicine. We thank Dr. Barton A. Kamen of South Western Medical School (Dallas, Tex) for the MOV19 antibody.

REFERENCES

1. Ferrari G, Rossini MS, Giavazzi R, et al. An *in vivo* model of somatic cell gene therapy for human severe combined immunodeficiency. *Science.* 1991;251:1363–1366.
2. Hanania EG, Fu S, Roninson I, Zu Z, Deisseroth A. Resistance to taxol chemotherapy produced in mouse marrow cells by safety-modified retroviruses containing a human MDR-1 transcription unit. *Gene Ther.* 1995;2:279–284.
3. Rosenfeld MA, Yoshimura K, Trapnell BC, et al. *In vivo* transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell.* 1992;68:143–155.
4. Brody SL, Jaffe HA, Han SG, Wersto RP, Crystal RG. Direct *in vivo* gene transfer and expression in malignant cells using adenovirus vectors. *Hum Gene Ther.* 1994;5: 437–447.
5. Hirschowitz EA, Ohwada A, Pascal WR, Russi TJ, Crystal RG. *In vivo* adenovirus-mediated gene transfer of the *Escherichia coli* cytosine deaminase gene to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. *Hum Gene Ther.* 1995;6:1055–1063.
6. Descamps V, Duffour MT, Mathieu MC, et al. Strategies for cancer gene therapy using adenoviral vectors. *J Mol Med.* 1996;74:183–189.
7. Bramson JL, Graham FL, Gauldie J. The use of adenoviral vectors for gene therapy and gene transfer *in vivo*. *Curr Opin Biotechnol.* 1995;6:590–595.
8. Zhang WW, Fang X, Mazur W, French BA, Georges RN, Roth JA. High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus. *Cancer Gene Ther.* 1994;1:5–13.
9. Leavitt J. Discovery and characterization of two novel human cancer-related proteins using two-dimensional gel electrophoresis. *Electrophoresis.* 1994;15:345–357.
10. Park T, Chen ZP, Leavitt J. Activation of the leukocyte plastin gene occurs in most human cancer cells. *Cancer Res.* 1994;54:1775–1781.
11. Lin CS, Park T, Chen ZP, Leavitt J. Human plastin genes. *J Biol Chem.* 1993;268:2781–2792.
12. Lin CS, Chen ZP, Park T, Ghosh K, Leavitt J. Characterization of the human L-plastin gene promoter in normal and neoplastic cells. *J Biol Chem.* 1993;268:2793–2801.
13. Zhang WW, Fang X, Branch CD, Mazur W, French BA, Roth JA. Generation and identification of recombinant adenovirus by liposome-mediated transfection and PCR analysis. *Biotechniques.* 1993;15:868–872.
14. Graham FL, Prevec L. Manipulation of adenovirus vectors. In: Murray EJ, ed. *Methods in Molecular Biology.* Clifton, NJ: The Human Press; 1991:109–128.
15. Roederer M, Fiering S, Herzenberg LA. FACS-gal: flow cytometric analysis and sorting of cells expressing reporter gene constructs. *Methods.* 1991;2:248–260.
16. Neering SJ, Hardy SF, Minamoto D, Spratt SK, Jordan CT. Transduction of primitive human hematopoietic cells with recombinant adenovirus vectors. *Blood.* 1996;88:1147–1155.
17. Clarke MF, Apel IJ, Benedict MA, et al. A recombinant bcl-x_s adenovirus selectively induces apoptosis in cancer cells but not in normal bone marrow cells. *Proc Natl Acad Sci USA.* 1995;92:11024–11028.
18. Seth P, Brinkmann U, Schwartz GH, et al. Adenovirus-mediated gene transfer to human breast tumor cells: an approach for cancer gene therapy and bone marrow purging. *Cancer Res.* 1996;56:1346–1351.
19. Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins $\alpha V\beta 3$ and $\alpha V\beta 5$ promote adenovirus internalization but not virus attachment. *Cell.* 1993;73:309–319.
20. Wickham TJ, Filardo EJ, Cheresh DA, Nemerow GR. Integrin $\alpha V\beta 5$ selectively promotes adenovirus-mediated cell membrane permeabilization. *J Cell Biol.* 1994;127:257–264.

[CANCER RESEARCH 61, 4405–4413, June 1, 2001]

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

Received 2/3/00; accepted 4/10/01.

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[1] Supported by BC980260 from the United States Army Breast Cancer Research Program, the Breast Cancer Women's League of Greenwich Connecticut, the Hull Development Fund of the Yale Cancer Center, the Bush Leukemia Research Fund, the American Cancer Society, The Audrey Demas Melanoma Research Fund, the Breast Cancer Research Foundation, and the Ensign Professorship in Medicine at Yale (to A. D.).

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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 $\alpha v\beta 3$ (LM609) and $\alpha v\beta 5$ (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 $\alpha v\beta 3$ (LM609) integrin and the $\alpha v\beta 5$ (P1F6) integrin and to the CAR receptors were used to detect the density of the human $\alpha v\beta 3$, $\alpha v\beta 5$, 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 $\alpha v\beta 3$ and $\alpha v\beta 5$ 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 $\alpha v\beta 3$ 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 $\alpha v\beta$ -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 $\alpha v\beta 5$ 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 $\alpha v\beta 3$ 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 $\alpha V\beta 5$ 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 $\alpha v\beta 3$ 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 $\alpha v\beta 3$ 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

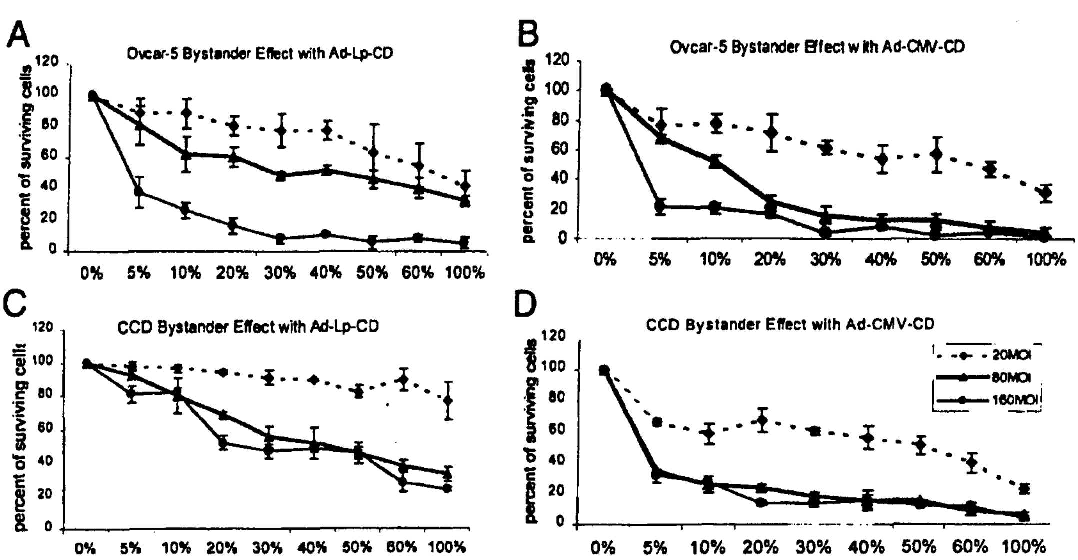

Fig. 1. Toxicity of vectors at varying levels of infected cells. Ovcar-5 (*A* and *B*) or CCD (*C* and *D*) cell lines were infected at varying MOI (20, 80, and 160) using the Ad-CMV-CD (*B* and *D*) or Ad-LP-CD (*A* and *C*) adenoviral vectors. The infected cells and noninfected cells were mixed in 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.

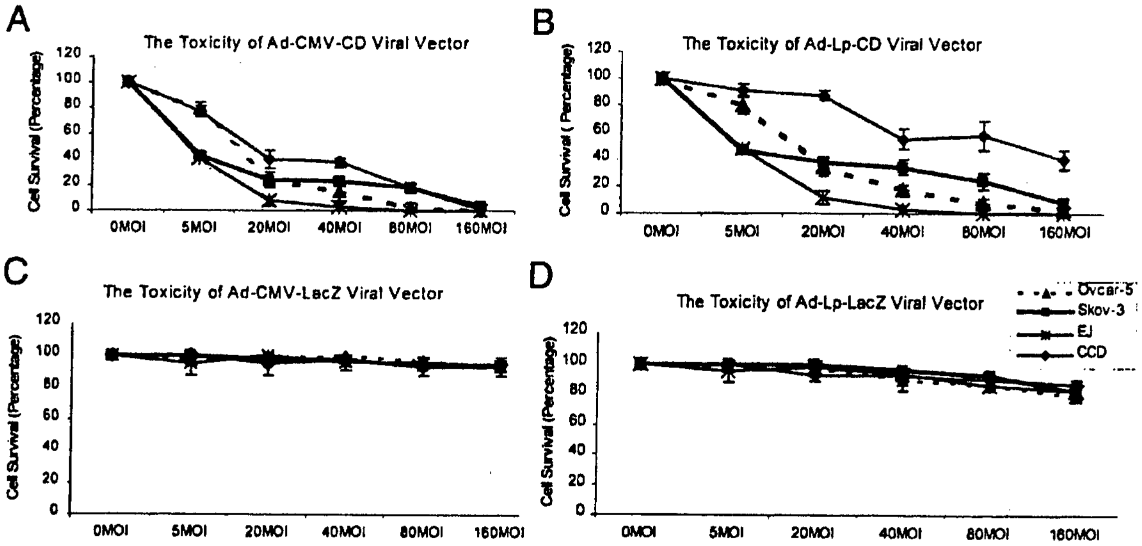

Fig. 2. Study of the toxicity of the control Lac-Z vector *versus* the CD vector. Cells (2×10^5) were infected at 0, 5, 20, 40, 80 and 160 MOI of vector with Ad-CMV-CD (*A*), Ad-Lp-CD (*B*), Ad-CMV-LacZ (*C*), Ad-Lp-LacZ (*D*) vectors for 90 min. Then cells were seeded in six-well plates in duplicate 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

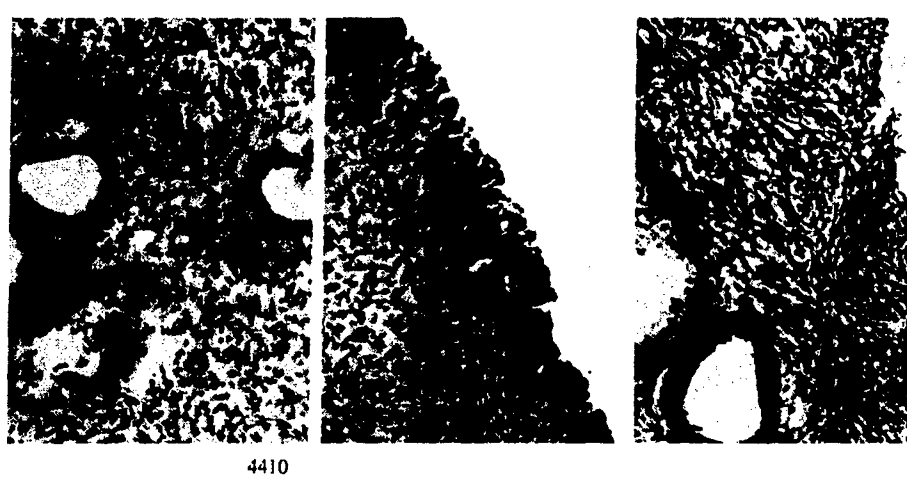

Fig. 3. Ovarian organ cultures. Normal ovarian tissue was obtained from patients undergoing abdominal surgical procedures. The tissues were cut into small pieces and cultured in DMEM:Ham's F12 medium with 10% charcoal-stripped serum. Twenty-four to forty-eight h later, the tissues were infected with 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 MOI 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

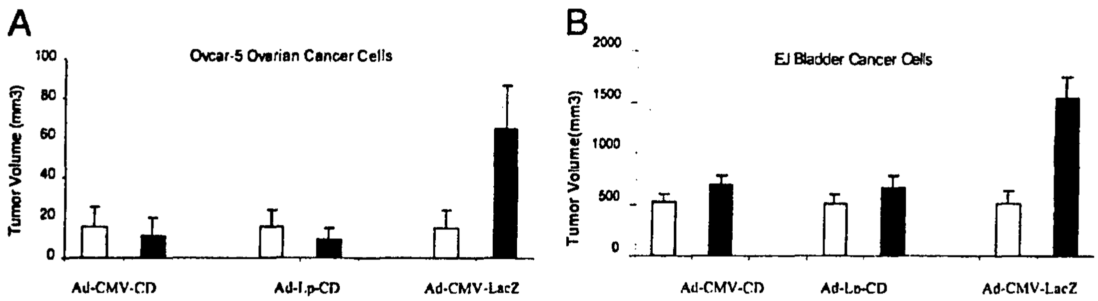

Fig. 4. Effect of *in vivo* injection of tumor nodules with adenoviral vectors. Ovcar-5 (*A*) or EJ (*B*) cells (5×10^6) were injected s.c. into nude mice. After 3 weeks, the tumor nodules were measured. Then, 10^8 pfu of the Ad-CMV-CD, 10^8 pfu of the Ad-Lp-CD, or 10^8 pfu of the Ad-CMV-LacZ vectors 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.

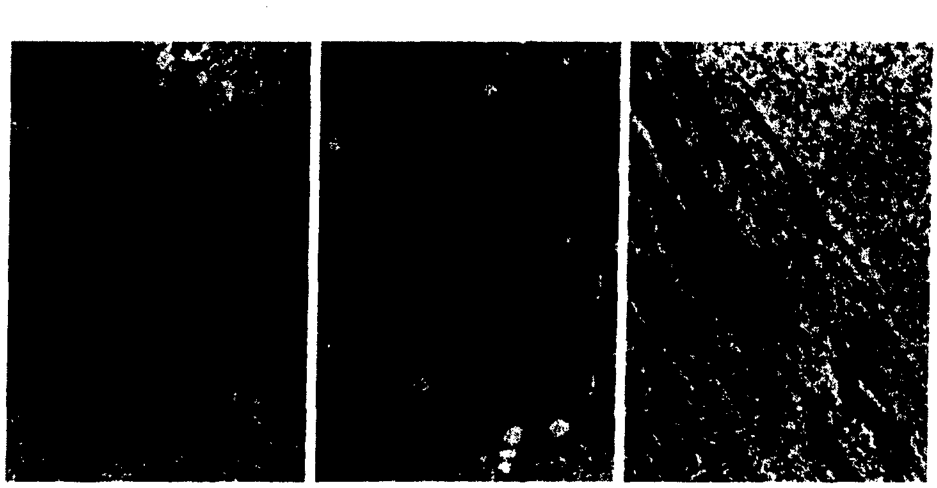

Fig. 5. Vector toxicity to tumor cells and adjacent tissues. Ovcar-5 cells (5×10^6) were injected s.c. After 3 weeks, 10^8 pfu of Ad-Lp-CD (*right*), 10^8 pfu of Ad-CMV-CD (*left*), or 10^8 pfu of Ad-CMV-LacZ (*middle*) vector were injected into each tumor nodule, and 500 mg/kg 5FC was given i.p. once 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.

REFERENCES

1. Descamps, V., Duffour, M. T., Mathieu, M. C., Ferbabdez, N., Cordier, L., Abina, M. A., Kremer, E., Perricaudet, M., and Haddada, H. Strategies for cancer gene therapy using adenoviral vectors. J. Mol. Med., *74:* 183-189, 1996.
2. Koyama, F., Sawada, H., Hirao, T., Fujii, H., Hamada, H., and Nakano, H. Combined suicide gene therapy for human colon cancer cells using adenovirus-mediated transfer of *Escherichia coli* cytosine deaminase gene and *Escherichia coli* uracil phosphoribosyltransferase gene with 5-fluorocytosine. Cancer Gene Ther., *7:* 1015-1022, 2000.
3. Xiang, J., Chen, Y., and Moyana, T. Combination immunotherapy for established tumors with engineered tumor vaccines and adenovirus-mediated gene transfer. Cancer Gene Ther., *7:* 1023–1033, 2000.
4. Leavitt, J. Discovery and characterization of two novel human cancer-related proteins using two-dimensional gel electrophoresis. Electrophoresis, *15:* 345–357, 1994.
5. Lin, C. S., Chang, C. H., and Huynh, T. The murine L-plastin gene promoter: identification and comparison with the human L-plastin gene promoter. DNA Cell Biol., *1:* 9–16, 1997.
6. Park, T., Chen, Z. P., and Leavitt, J. Activation of the leukocyte plastin gene occurs in most human cancer cells. Cancer Res., *54:* 1775-1781, 1994.
7. Chung, I., Peter, E. S., Crystal, R. G., Pizzorno, G., and Deisseroth, A. Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cell. Cancer Gene Ther., *6:* 99–106, 1999.
8. Hirschowitz, E. A., Ohwada, A., Pascal, W. R., Russi, T. J., and Crystal, R. G. *In vivo* adenovirus-mediated gene transfer of the *Escherichia coli* cytosine deaminase gene to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. Hum. Gene Ther., *6:* 1055–1063, 1995.
9. Tong, X., Engehausen, D. G., Freund, C. T., Agoulnik, I., Guo, Z., Oehler, M. K., Kim, T. E., Hasenburg, A., and Kieback, D. G. The efficacy of adenovirus-mediated gene therapy of ovarian cancer is enhanced by using the cytomegalovirus promoter. Anticancer Res., *18:* 719–725,1998.
10. Graham, F. L., and Prevec, L. Manipulation of adenovirus vectors. *In:* E. J. Murray (ed.), Methods in Molecular Biology, Vol. 7, pp. 109-128. Clifton, NJ: The Human Press Inc., 1991.
11. Neering, S. J., Hardy, S. F., Minamoto, D., Spratt, S. K., and Jordan, C. T. Transduction of primitive human hematopoietic cells with recombinant adenovirus vectors. Blood, *88:* 1147–1155, 1996.
12. Couffinhal, T., Kearney, M., Sullivan, A., Silver, M., and Isner, J. M. Histochemical staining following *LacZ* gene transfer underestimates transfection efficiency. Hum. Gene Ther., *8:* 929–934, 1997.
13. Theodore, L., Rehemtulla, A., Emily, Y., Trosko, J. E., and Stetson, P. L. Preferential cytotoxicity of cells transduced with cytosine deaminase compared to bystander cells after treatment with 5-flucytosine. Cancer Res., *58:* 2588–2593, 1998.
14. Kianmanesh, A. R., Perrin, H., Panis, Y., Houssin, D., and Klatzmann, D. A. "Distant" bystander effect of suicide gene therapy: regression of nontransduced tumors together with a distant transduced tumor. Hum. Gene Ther., *8:* 1807-1814, 1997.
15. Ramesh, R., Marrogi, A. J., Munshi, A., and Freeman, S. M. Potentiation of the bystander effect by immunization combined with suicide gene therapy. Adv. Exp. Med. Biol., *451:* 125–131, 1998.
16. Parsels, L. A., Kowalski, M. P., Wagner, L. M., Parsels, J. D., Maley, F., and Maybaum, J. *Escherichia coli* thymidylate synthase expression protects human cells

from the cytotoxic effects of 5-fluorodeoxyuridine more effectively than human thymidylate synthase overexpression. Cancer Gene Ther., *7:* 1179–1187, 2000.
17. Sapi, E., Flick, M. B., and Rodov, S. Expression of CSF-1 and CSF-1 receptor by normal lactating mammary epithelial cells. J. Soc. Gynecol. Investig., *5:* 94–101, 1998.
18. Kelly, L., Molpus, D. K., Leonard, A., and Hasan, T., Characterization of a xenograft model of human ovarian carcinoma which produces intraperitoneal carcinomatosis and metastases in mice. Int. J. Cancer, *67:* 588–595, 1996.
19. Hwang, E. S., Kim, J. S., You, E. H., Lee, S. H., and Lee, J. H. Intraperitoneal gene therapy with adenoviral-mediated *p53* tumor suppressor gene for ovarian cancer model in nude mouse. Cancer Gene Ther., *6:* 172–178, 1999.
20. Pang, S. Targeting and eradicating cancer cells by a prostate-specific vector carrying the diphtheria toxin A gene. Cancer Gene Ther., *7:* 991–996, 2000.
21. Martin, D. S., Stolfi, R. L., Sawyer, R. C., and Yang, C. W. Therapeutic utility of utilizing low doses of *N*-(phosphonacetyl)-L-aspartic acid in combination with 5-fluorouracil: a murine study with clinical relevance. Cancer Res., *43:* 2317–2321, 1983.
22. Bergelson, J. B., Krithivas, A., Celi, L., Wickham, T., Crowell, R. L., and Fingerg, R. W. The murine CAR homolog is a receptor for coxsackie B viruses and adenoviruses. J. Virol., *1:* 415–419, 1998.
23. Bergelson, J. M., Cunningham, J. A., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. Science (Wash. DC), *275:* 1320–1323, 1997.
24. Roelvink, P. W., Lizonova, A., Lee, J. M., Li, Y., Kovesdi, I., and Wickham, T. J. The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. J. Virol., *10:* 7909–7915, 1998.
25. Garcia-Sanchez, F., Fu, S. Q., Nanakorn, T., Leffert, J. J., Cote, R., Crystal, R., and Deisseroth, A. Cytosine deaminase adenoviral vector and 5-fluorocytosin selectively reduce breast cancer cells 1 million-fold when they contaminate hematopoietic cells: a potential purging method for autologous transplantation. Blood, *92:* 672–682, 1998.
26. Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. Integrins avb3 and avb5 promoter adenovirus internalization but not virus attachment. Cell, *73:* 309–319, 1994.
27. Wickham, T. J., Filardo, E. J., Cheresh, D. A., and Nemerow, G. R. Integrin avb5 selectively promotes adenovirus mediated cell membrane permeabilization. J. Cell Biol., *127:* 257–264, 1993.
28. Roederer, M., Fiering, S., and Herzenberg, L. A. FACS-gal: flow cytometric analysis and sorting of cells expressing reporter gene constructs. Methods, *2:* 248–260, 1991.

doi:10.1006/mthe.2002.0680, available online at http://www.idealibrary.com on IDEAL

Adenoviral Vectors with E1A Regulated by Tumor-Specific Promoters Are Selectively Cytolytic for Breast Cancer and Melanoma

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We have previously demonstrated that a truncated form of the L-plastin promoter can confer tumor-specific patterns of expression on replication-incompetent adenoviral vector reporter and therapeutic transcription units. In this report, a 2.5-kb truncated version of the L-plastin promoter was placed 5′ to the E1A gene of a wild-type adenovirus. The vector generated (Ad-Lp-E1A) was directly cytotoxic to established breast and ovarian cancer cell lines and to primary explant cultures derived from ovarian cancer, but was not cytotoxic to explant cultures of normal mammary epithelial cells. This vector was not cytotoxic to cell lines in which the L-plastin E1A transcription unit was not expressed, whereas the same cell lines were sensitive to the cytotoxic effect of a replication-competent adenoviral vector in which the cytomegalovirus (CMV) promoter drove E1A expression. When the tyrosinase promoter/enhancer was placed 5′ to the E1A gene in the adenoviral backbone, the resulting vector (Ad-Tyr-E1A) was selectively toxic to melanoma cells and one percent as toxic to explants of ovarian cancer cells as the Ad-Lp-E1A vector. Injection of these vectors (Ad-Lp-E1A and Ad-Tyr-E1A) into nodules derived from the MCF-7 and MDA-MB-468 human breast cancer cell lines and the TF-2 human melanoma cell line, respectively, which were growing subcutaneously in severe combined immunodeficiency (SCID) mice, induced regression of these tumors. Such vectors may therefore be useful in cancer treatment.

Key Words: adenovirus, breast cancer, ovarian cancer, melanoma, E1A, L-plastin

INTRODUCTION

Recombinant adenoviral vectors have been widely used in preclinical models for *in vitro* and *in vivo* gene transfer. Adenoviral vector-mediated therapeutic gene expression has been achieved in a broad spectrum of eukaryotic cells and is independent of cell replication [1,2]. In addition, the E1 gene-deleted, replication-defective adenoviral vectors can accommodate large DNA inserts. Tissue-specific promoters have been used to regulate the expression of therapeutic transgenes in these vectors to restrict the toxicity of the treatment to the tissue of origin of the cancer [3]. One of the problems is the low percentage of cells in a tumor nodule that eventually becomes infected by replication-incompetent adenoviral vectors. Tissue-specific promoters or enhancers have also been used to regulate the expression of viral genes that are necessary to the replication of adenoviral vectors, as well as herpes simplex recombinant vectors to increase the number of cancer cells infected by the vector [4,5]. In this context, the minimal promoter/enhancer from the prostate-specific antigen (PSA) gene has been used to drive E1A expression and to create an adenovirus, designated CN706, that selectively replicates in PSA-positive cells [4]. A similar strategy using the albumin promoter has been used to develop a herpes simplex virus that selectively replicates in hepatoma cells [5]. Genetic complementation between mutations introduced into the E1B gene of the adenoviral vector and mutations in tumor suppressor genes in the tumor cells have been used to create adenoviral vectors that are conditionally replication-competent in the cancer cells but not in the normal tissues of the host [6].

We have been studying the feasibility of using a truncated form of the L-plastin promoter to create adenoviral vectors that selectively replicate within tumor cells and, therefore, are selectively toxic to these cells. The plastins constitute a family of human actin-binding proteins (isoforms) that are abundantly expressed in all normal

1525-0016/02 $35.00

doi:10.1006/mthe.2002.0680, available online at http://www.idealibrary.com on IDEAL

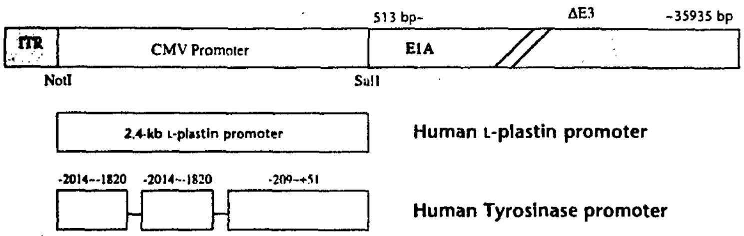

FIG. 1. Organization of conditionally replication-competent adenoviral vectors. The 2.5-kb L-plastin promoter or the human tyrosinase promoter and tissue-specific enhancer were inserted 5′ to the E1A and E1B genes. The regulatory elements left in the promoter in the adenoviral vectors are identical to those reported by Rodriguez *et al.* [4]. The viral packaging signal, which overlaps the E1A enhancer, has also been left in the area 5′ to the E1A transcription unit. Finally, it should be emphasized that both the E1A and E1B genes have been left in the vector.

replicating mammalian cells. One isoform, L-plastin, is constitutively expressed at high levels in mature hematopoietic cell types but is expressed in no other normal tissue. L-Plastin is, however, constitutively synthesized in most types of malignant human cells, suggesting that its expression is induced during tumorigenesis. L-Plastin expression is especially high in cancers that arise from estrogen-dependent tissues [7–10]. To test the feasibility of conferring tumor-specific conditional replication competency on the wild-type adenovirus, we have placed a 2.5-kb truncated form of the L-plastin promoter (Fig. 1) 5′ to the E1A gene (Ad-Lp-E1A). For comparison, we have also placed either the CMV promter (Ad-CMV-E1A) or the tyrosinase promoter/enhancer (Fig. 1) 5′ to the E1A gene in a wild-type adenovirus. Tyrosinase, which is the product of the albino locus and is a pigment cell-restricted enzyme that catalyzes the rate-limiting step in melanin synthesis, is highly expressed in melanoma cells. The tyrosinase promoter/enhancer cassette used for this purpose has been studied in human and mouse cells [11–13] for its ability to govern the expression of heterologous genes in the adenoviral vector.

The Ad-Lp-E1A vector is toxic to the L-plastin-positive MCF-7 and MDA-MB-468 established human breast cancer cell lines, as well as to explants of human ovarian cancer cells *in vitro,* but is not toxic to explant cultures of normal mammary epithelial cells. When injected intratumorally, the Ad-Lp-E1A vector suppresses the growth of L-plastin-positive breast cancer cells in severe combined immunodeficiency (SCID) mice. We also demonstrate that the vector that contains the tyrosinase promoter/enhancer cassette driving the E1A gene (Ad-Tyr-E1A) suppresses the growth of the TF-2 human melanoma cell line in SCID mice. The results demonstrate that the Ad-Lp-E1A vector in which E1A gene expression is regulated by a truncated L-plastin promoter is 100 times as toxic to ovarian cancer cells as the Ad-Tyr-E1A vector, which itself is toxic to melanoma cell lines. Our results also suggested that a correlation exists between the ability of cancer cell lines to support the expression of the E1A gene following exposure to the Ad-Lp-E1A vector, and the degree of detachment and lysis of the exposed cancer cells.

RESULTS

Construction of Conditionally Replication-Competent Adenoviral Vectors

To construct replication-competent adenoviral vectors, we inserted the 2.5-kb L-plastin promoter *ScaI* fragment or the cytomegalovirus (CMV) promoter or the tyrosinase promoter/enhancer 5′ to the E1A gene of the wild-type adenovirus (Fig. 1) according to the reported method [14]. After adenoviral vector particles were generated in HEK 293 cells, the vector DNA was purified. The presence and structure of the promoter in each adenoviral vector was confirmed by PCR, by restriction enzyme analysis, and by DNA sequencing of the promoter in the AD5 sequence.

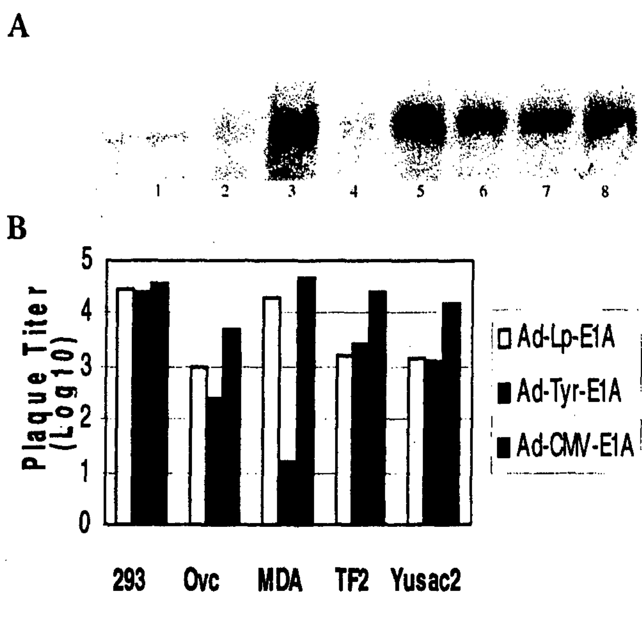

FIG. 2. Analysis of E1A expression and function. (A) E1A protein production in cells infected with Ad-Lp-E1A (lanes 1–4) and Ad-CMV-E1A (lanes 5–8). Ad5E1A protein expression in Yusac2 human melanoma cell line (lanes 1 and 5), TF2 human melanoma cell line (lanes 2 and 6), MDA-MB-468 human breast cancer cell line (lanes 3 and 7), and normal human mammary epithelial cells (lanes 4 and 8) was detected by western blot analysis. The molecular weight of the E1A protein marker is 46 kDa. We added 10 μg of protein to each lane. (B) The replication of the viral vectors (Ad-Lp-E1A, Ad-Tyr-E1A, and Ad-CMV-E1A) in different cell lines (293, Ovcar-5, MDA-MB-486, TF2, and Yusac2). Cell monolayers (2×10^5 cells/well) in six-well plates were inoculated with 2×10^5 pfu of the adenoviral vectors. The determination of the yield of vector (plaque titer) was carried out 72 hours after infection by the plaque assay on 293 cells in six-well plates.

doi:10.1006/mthe.2002.0680, available online at http://www.idealibrary.com on IDEAL

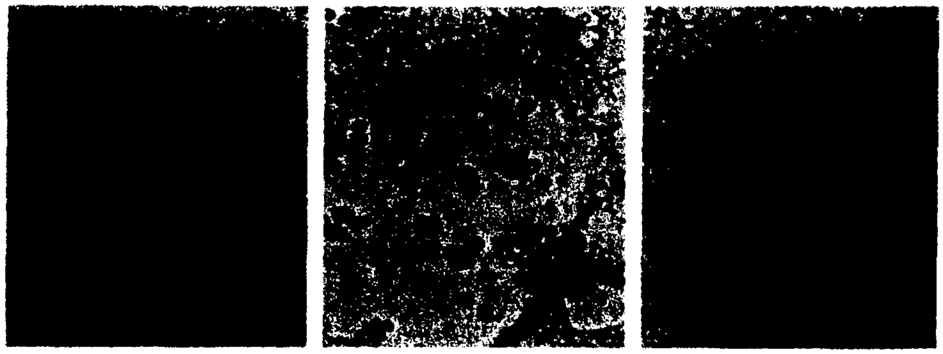

FIG. 3. Study of the sensitivity of breast cancer cells to the cytotoxic effect of the Ad-Lp-E1A vector. The MDA-MB-468 breast cancer cell line was exposed to the Ad-Lp-E1A vector at an MOI of 100 (left) and at an MOI of 10 (middle). The cells in the right panel were not exposed to vector. The pictures were taken 9 days after infection.

Western Blot Analysis of E1A Expression

E1A protein production in cells infected with Ad-Lp-E1A (Fig. 2A, lanes 1–4) and in those infected with Ad-CMV-E1A, as defined in Fig. 1, (Fig. 2A, lanes 5–8) was determined by western blot analysis. The Ad-CMV-E1A adenoviral vector produced considerable E1A protein in the Yusac2 and TF2 human melanoma cell lines and in the MDA-MB-468 human breast cancer cell line (Fig. 2A, lanes 5–7). Although the Ad-Lp-E1A produced a high level of E1A protein in the MDA-MB-468 established breast cancer cell line (Fig. 2A, lane 3), it produced a much lower level of expression in subconfluent explant cultures of normal human mammary epithelial cells (Fig. 2A, lane 4). These data show that the L-plastin promoter can drive the expression of the E1A gene in breast cancer cells but not in explant cultures of normal human mammary epithelial cells, suggesting a tumor-specific pattern of expression. The L-plastin promoter in Ad-Lp-E1A produces a much lower level of E1A expression in the TF-2 human melanoma cell line (Fig. 2A. lane 2) than in the MDA-MB breast cancer cell line. This suggests a tissue-specific pattern of expression of these vectors as well. The fact that the Ad-CMV-E1A vector produced high levels of E1A expression in all of the cell lines tested (Fig. 2A, lanes 5–8), even the ones in which the Ad-Lp-E1A and Ad-Tyr-E1A vectors produced only very low or undetectable levels of E1A expression, suggests that the low levels of expression in certain cell lines following exposure to the Ad-Lp-E1A and Ad-Tyr-E1A vectors was not due to difficulty in infecting the target cell lines but due to the low strength of the promoters in these cell lines.

The replication of the viral vectors Ad-Lp-E1A, Ad-Tyr-E1A, and Ad-CMV-E1A in different cell lines (293, Ovcar-5, MDA-MB-486, TF2, and Yusac2) was determined by assaying the replicated virus yield of the vectors in those cell lines (Fig. 2B). The replication of the Ad-Lp-E1A and Ad-CMV-E1A vectors is equivalent in the MDA-MB-468 human breast cancer cell line. The Ad-Tyr-E1A vector can replicate in the TF2 and Yusac2 human melanoma cell lines, but replicates much less in the MDA-MB-468 breast cancer cell line. These data suggest that the replication of these vectors will exhibit tissue and tumor-specific patterns, in agreement with the E1A expression data presented in Fig. 2A.

Cytolysis Assays in Cell Lines and Explant Cultures of Tumor Cells Following Exposure to Viral Vectors

To test if the replication of the Ad-Lp-E1A and Ad-Tyr-E1A vectors results in a direct cytotoxic effect, these vectors were analyzed for their ability to generate rounding up and detachment of established human cancer cell lines and explant cultures of human tumor cells derived from excised ovarian cancer tissue from patients. The MDA-MB-486 human breast cancer cell line, which has been shown in our laboratory to support the expression

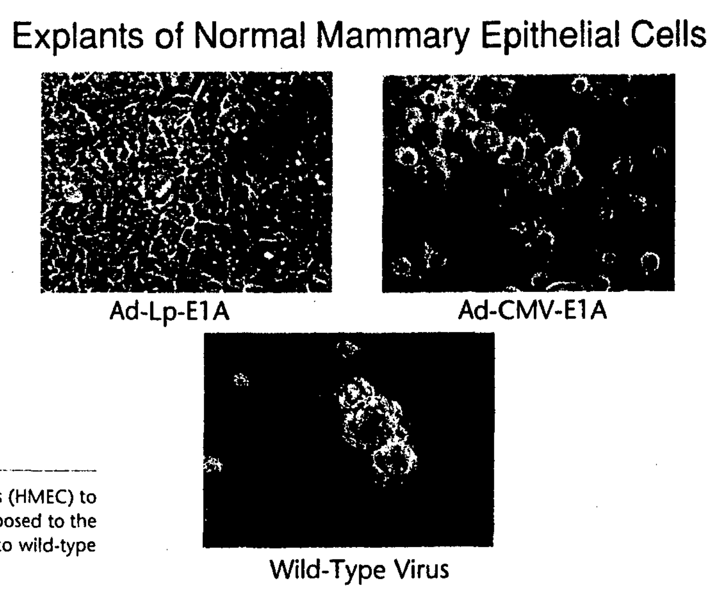

FIG. 4. Study of the sensitivity of normal human mammary epithelial cells (HMEC) to the cytotoxic effect of the Ad-Lp-E1A vector. Normal HMEC cells were exposed to the Ad-Lp-E1A vector (top left), to the Ad-CMV-E1A vector (top right), and to wild-type adenovirus (bottom) at an MOI of 10 for 9 days.

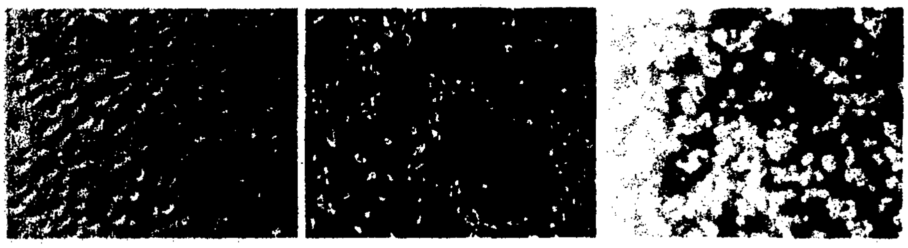

FIG. 5. Study of the sensitivity of explant cultures of human ovarian cancer cells to the cytotoxic effect of the Ad-Lp-E1A vector 9 days after infection. Explant cultures of human ovarian cancer cells were exposed to the Ad-Lp-E1A vector at MOIs of 1 and 10 (middle and right, respectively) or were not exposed to the vector (left).

of genes regulated by the L-plastin promoter [7,15] and has been shown in this work to support the expression of the E1A gene when exposed to the Ad-Lp-E1A vector (Fig. 2A, lane 3), was used to test the lytic effect of the Ad-Lp-E1A vector *in vitro*. The MDA-MB-468 cells were infected with Ad-Lp-E1A at different MOIs. Complete lysis of the cells was observed 9 days after infection at an MOI of 10 (Fig. 3 middle). Another breast cancer cell line, MCF-7, produced the same result (data not shown).

Normal human mammary epithelial cells, which showed only very low expression levels of the E1A gene following exposure to the Ad-Lp-E1A vector (Fig. 2A, lane 4) but showed E1A expression following exposure to the Ad-CMV-E1A adenoviral vector (Fig. 2A, lane 8), were exposed to the Ad-Lp-E1A and the Ad-CMV-E1A vectors and to the wild-type adenovirus at an MOI of 10. A rounding up and lifting off of the cells was observed following exposure of the normal human mammary epithelial cells to the Ad-CMV-E1A vector or to the wild-type adenovirus after 9 days (Fig. 4). In this experiment, no lysis was seen following exposure of the normal human mammary epithelial cells to the Ad-Lp-E1A vector (Fig. 4). Thus, the results in Figs. 2–4 show that there is a correlation between the expression of the E1A gene, the replication of the vector, and the lysis of the cell lines following exposure to the Ad-Lp-E1A vector. These results suggest that Ad-Lp-E1A vector can replicate only in cells that support the expression of genes regulated by the L-plastin promoter.

To test for a direct cytolytic effect of the Ad-Lp-E1A vector in human ovarian carcinoma cells, explants of ovarian cancer cells excised from patients were cultured as monolayers and then exposed to the Ad-Lp-E1A vector. At MOIs of 1 and 10, a remarkable lytic effect was observed in the explant cultures of ovarian carcinoma (Fig. 5). Then, the effect of the Ad-Lp-E1A, Ad-Tyr-E1A, and Ad-CMV-E1A vectors and the negative control Ad-CMV-LacZ adenoviral vector on explant cultures of human ovarian carcinoma cells from a patient sample was tested at different MOIs (Fig. 6). The difference in the IC50 between the Ad-Lp-E1A and Ad-Tyr-E1A vectors is about 100-fold (Fig. 6). The difference in the IC50 between the Ad-Lp-E1A and the Ad-CMV-LacZ vectors was 1000-fold. In addition, the data show that the cytolytic effect of the Ad-Lp-E1A vector is equivalent to that of the Ad-CMV-E1A vector.

When we infected the TF-2 human melanoma cell line with the Ad-Lp-E1A, Ad-Tyr-E1A, and Ad-CMV-E1A vectors, as well as with the wild-type adenovirus, the cultures exposed to the Ad-CMV-E1A and Ad-Tyr-E1A vectors, as well as the wild-type adenovirus 5, showed lytic effects. In contrast, cultures of the TF-2 human melanoma cell line exposed to the Ad-Lp-E1A vector did not show detectable cytotoxicity (Fig. 7). These data show that the toxic effect of the Ad-Lp-E1A vector is much lower in the TF2 human melanoma cell line than in the ovarian or breast cancer cell lines. This suggests that the L-plastin promoter, when placed 5′ to the E1A gene in the adenoviral vector, can act as a tissue-specific, as well as tumor-specific, promoter.

Treatment of Human Breast Cancer Xenograft with the Ad-Lp-E1A Vector and of Human Melanoma Xenograft with the Ad-Tyr-E1A Vector

To evaluate the therapeutic efficacy of the Ad-Lp-E1A vector *in vivo*, MCF-7 and MDA-MB-468 human breast cancer

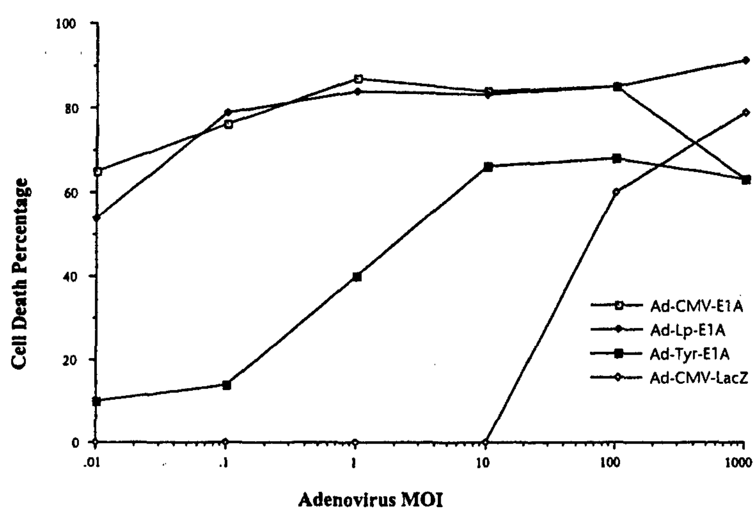

FIG. 6. Study of the sensitivity of explant cultures of ovarian cancer cells to adenoviral vectors. Monolayer explant cultures of ovarian cancer cells were exposed to varying MOIs of the Ad-Lp-E1A vector, of the Ad-CMV-E1A vector, of the Ad-Tyr-E1A vectors, and of the replication-incompetent Ad-CMV-LacZ negative control vector. The percentage of cells killed was measured by the MTT assay [15] 7 days after infection. The percentage of cells killed is calculated as the ratio of the absorbance at 490 nm in the test culture to that of the culture without vector added. All data are the result of triplicate assays, and the standard deviation is less than 15%.

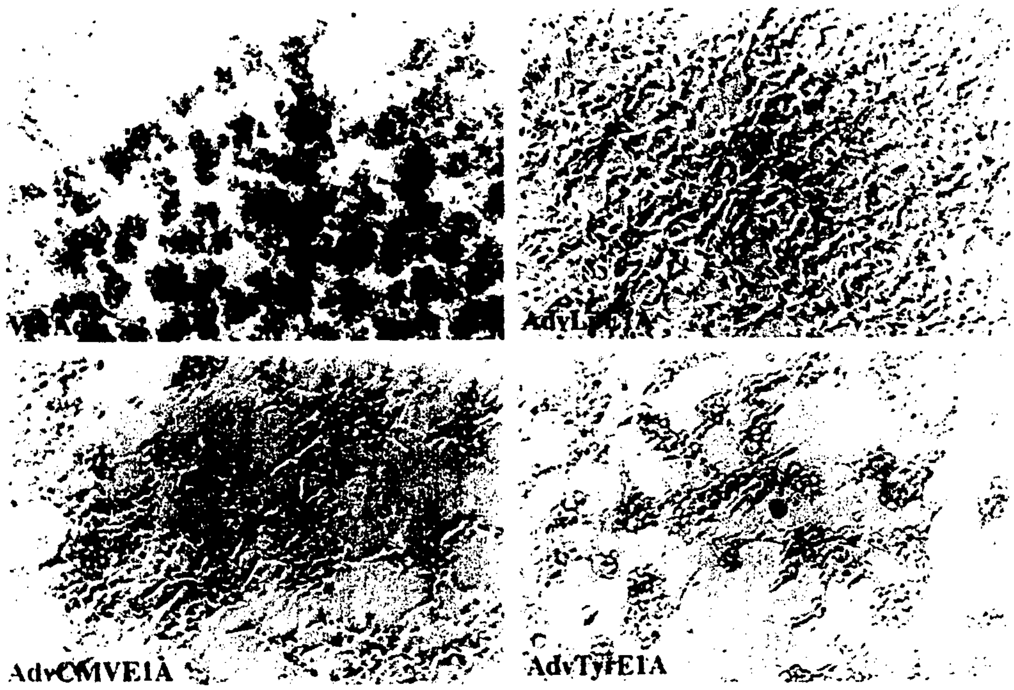

FIG. 7. Cytotoxicity of the adenoviral vectors to the melanoma TF-2 cells 9 days after infection. Monolayers of the human melanoma cell line TF-2 were exposed to wild-type virus (top left), the Ad-CMV-E1A vector (bottom left), the Ad-Lp-E1A vector (top right), and the Ad-Tyr-E1A vector (bottom right).

cell lines were injected subcutaneously into SCID mice, allowed to grow into subcutaneous tumor nodules of at least 50 mm^3 in size, and injected three times with 1×10^8 pfu of the wild-type adenovirus (Fig. 8B), the Ad-Lp-E1A vector (Fig. 8B), or the Ad-CMV-LacZ vector (Fig. 8B), once every 2 days. Injection with the Ad-Lp-E1A vector was associated with inhibition of MCF-7 tumor cell growth (Figs. 8A and 8B). The standard deviations about the mean are defined for each data point by the vertical error bars. In Fig. 8B, the standard deviation about the mean of the sizes of the tumor nodules in animals injected intratumorally with the wild-type adenovirus or the Ad-Lp-E1A vector do not overlap with the standard deviation about the mean of the tumor sizes in animals injected intratumorally with PBS or with the Ad-CMV-LacZ vector. The differences between the standard deviation about the mean of the tumor nodule size following intratumoral injection with the Ad-Lp-E1A vector and the standard deviation about the mean of the tumor nodule size following intratumoral injection with the Ad-CMV-LacZ vector or PBS are statistically significantly different at the $P < 0.0001$ level, using the unpaired *t*-test. If one tests whether the means of the sizes of the tumor nodules injected with the Ad-Lp-E1A vector are different from the means of the sizes of the tumor nodules injected with the Ad-CMV-LacZ vector or PBS using the upaired *t*-test, the two-tailed *P* value is 0.0152 or 0.0262, respectively, which is considered significant. These results indicate that injection of the Ad-Lp-E1A vector can suppress the growth of the MCF-7 cell line *in vivo*. A similar result was observed in MDA-MB-468 human breast cancer cell line xenografts (Fig. 8C). However, all injected cell lines eventually regrew in this model.

In a similar manner, the human melanoma TF-2 cell line was injected subcutaneously and allowed to grow into a tumor nodule in SCID mice to test the lytic effect of the Ad-Tyr-E1A vector on human melanoma cells *in vivo*. Then, these nodules were injected intratumorally once with 1×10^8 total particles of the vectors Ad-Tyr-E1A (Fig. 9), wild-type adenovirus (Fig. 9), and Ad-CMV-LacZ (Fig. 9), and with PBS (Fig. 9). The injection of the wild-type adenovirus into the TF-2 tumor nodules, or the injection of the Ad-Tyr-E1A vector into the tumor nodules, resulted in a reduction in the size of the melanoma tumor nodule, whereas no reduction in tumor growth occurred with injection of either PBS or the Ad-CMV-LacZ vector (Fig. 9). The standard deviations about the mean of the sizes of the tumor nodules injected intratumorally with the wild-type adenovirus or the Ad-Tyr-E1A vector were less than and did not overlap with the standard deviation about the mean of the tumor nodule sizes in animals injected intratumorally with PBS or with the Ad-CMV-LacZ vector. Although the TF-2 tumor nodules injected with the Ad-Tyr-E1A vector appeared to be growing more slowly than the TF-2 nodules injected with PBS or with the AD-CMV-LacZ vector, the differences in the standard deviations of the means of the tumor sizes injected with Ad-Tyr-E1A versus PBS or Ad-CMV-LacZ were not statistically significantly different (*P* values 0.1178 or 0.1599, respectively), as determined by the unpaired *t*-test. This lack of statistical significance may reflect the wide range of replicate values in the PBS- and Ad-CMV-LacZ-injected tumor nodules. Longer follow-up showed that the wild-type adenovirus- or Ad-Tyr-E1A-vector injected TF-2 human melanoma cell line eventually regrew.

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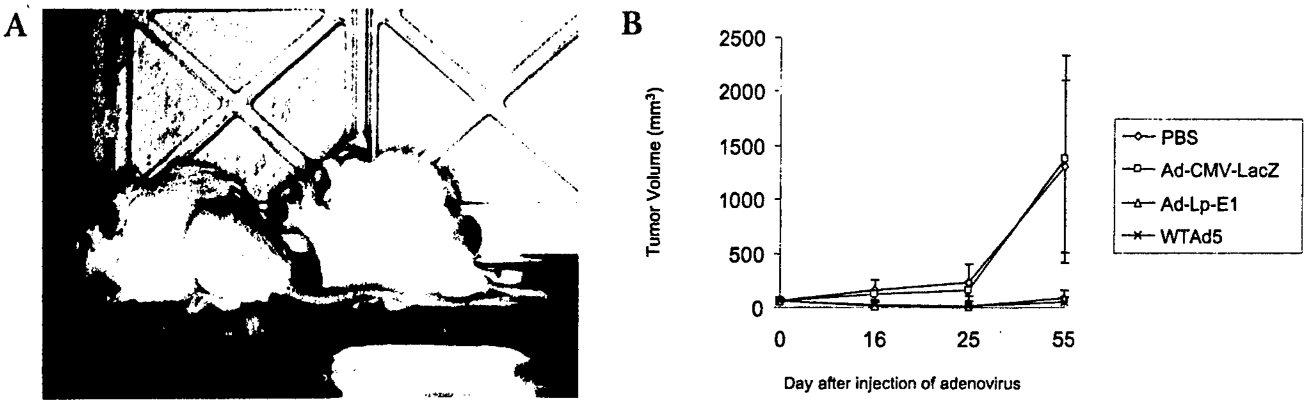

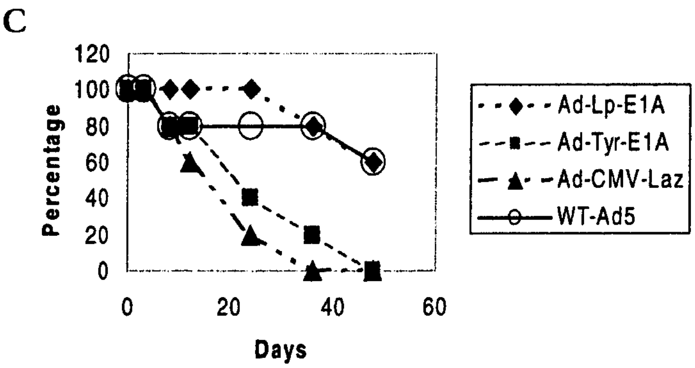

FIG. 8. The treatment breast cancer xenografts with adenoviral vectors. (A) Photograph of mice injected intratumorally with either the Ad-Lp-E1A vector (left) or with the Ad-CMV-LacZ vector (right) following injection of MCF-7 breast cancer cells. (B) The treatment of MCF-7 breast cancer xenografts with recombinant adenovirus. Tumor xenografts were treated with the recombinant vectors (10^8 pfu) Ad-Lp-E1A, wild-type adenovirus, and Ad-CMV-LacZ, and with PBS buffer by intratumoral injection on day 0 (1 week after tumor subcutaneous incubation) and measured. The data represent the mean of five replicate animals. The bars indicate the standard deviation about the mean of these values. The standard deviations about the mean (vertical error bars) of the sizes of the tumor nodules in animals injected with the negative controls (PBS or Ad-CMV-LacZ) and the test vectors (Ad-Lp-E1A or wild-type adenovirus) are statistically significantly different at the 0.0001 level, by the unpaired *t*-test. (C) The survival analysis of SCID mice with MDA-MB-486 xenografts. To establish subcutaneous tumor nodules, mice were inoculated subcutaneously in the rear flank with 5×10^6 MDA-MB-468 cells in 100 μl of PBS. The tumor growth was assessed by measuring bidimensional perpendicular diameters. Each animal was injected intratumorally with 10^9 pfu of the following viral vectors 1 week after inoculation of the MDA-MB-468 breast cancer cells: Ad-Lp-E1A, Ad-Tyr-E1A, wild-type adenovirus, and Ad-CMV-LacZ. The animals were killed by isoflurane when the tumors reached a volume of > 1000 mm³.

DISCUSSION

The goal of this work was to create adenoviral vectors that displayed tumor-specific replication competency and that were directly cytotoxic to tumor cells. In these replication-competent adenoviral vectors, we kept E1A and E1B intact because deletion of the E1B gene or the reduction of the expression of both the E1A and E1B genes through the use of an inducible promoter will decrease remarkably the replication of these vectors [16]. In the vectors described in this paper, the E1A promoter region was deleted, but the enhancer that overlaps the 5′ end of the viral packaging signal remains. This remaining viral element may enhance the L-plastin promoter, because the L-plastin promoter in the vectors we have developed appears to be stronger in the adenoviral vector than in plasmid expression vectors (data not shown). The L-plastin promoter is a tissue-specific but weak promoter.

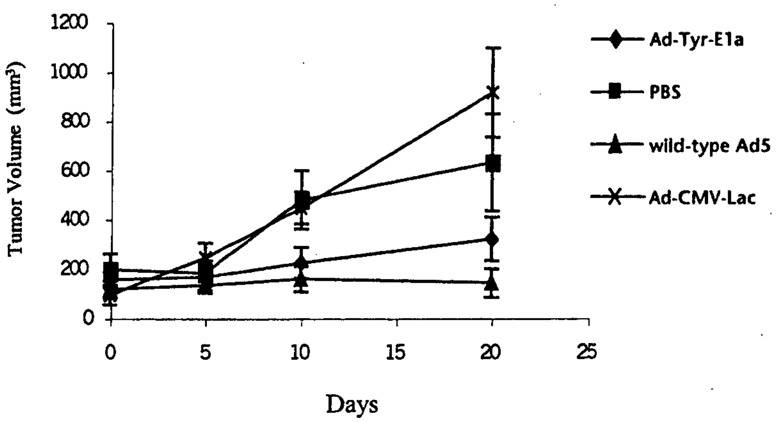

FIG. 9. The treatment of TF-2 melanoma tumor xenografts with recombinant adenoviral vectors. The tumor xenografts were injected intratumorally with 10^8 pfu of Ad-Tyr-E1A, wild-type adenovirus, or Ad-CMV-LacZ, or by PBS buffer, by intratumoral injection on day 0 (1 week after subcutaneous tumor cell inoculation) and measured weekly. The data represent the mean of five replicate animals. The bars represent the standard deviation about the mean. The standard deviations about the mean of the tumor sizes in animals injected with either PBS or Ad-CMV-LacZ were not found to be statistically significantly different with the standard deviation about the mean of the sizes of tumor nodules in animals injected with the Ad-Tyr-E1A vector ($P = 0.1178$ or 0.1599) by the unpaired *t*-test.

doi:10.1006/mthe.2002.0680, available online at http://www.idealibrary.com on IDEAL

We chose to compare the cytotoxic action of replication-competent vectors, which contained the L-plastin promoter 5′ to the E1A gene (Ad-Lp-E1A), with another vector that contained the tyrosinase promoter and tissue-specific enhancer 5′ to the E1A gene (Ad-Tyr-E1A), with a vector in which the CMV promoter drove the E1A gene (Ad-CMV-E1A), and with the wild-type adenovirus. These experiments extended previous work in our laboratory with replication-incompetent adenoviral vectors, which showed that a truncated form of the L-plastin promoter was active in established neoplastic cell lines derived from estrogen-dependent tissues, even after being embedded in the adenoviral backbone [7,15].

We showed first that the Lp-E1A transcription unit of the Ad-Lp-E1A vector was expressed in breast cancer cell lines but not in explant cultures of normal human mammary epithelial cells. We showed as well that the tyrosinase-E1A transcription unit was not active in breast cancer cell lines. We then showed that the Ad-Lp-E1A was cytotoxic *in vitro* to the MCF-7 and the MDA-MB-468 established human breast cancer cell lines, destroying the entire monolayer at an MOI of 10, but was not toxic to normal human mammary epithelial cells at the same MOI. The Ad-Lp-E1A also caused lifting up and rounding up of all of the cells in explant cultures of ovarian cancer cells obtained from surgical specimens when the vector was added at an MOI of 10. This pattern of toxicity (Figs. 3–5), which mirrored the expression of the E1A transcription unit when placed under control of the L-plastin promoter (Fig. 2A), suggests that the L-plastin promoter is specifically active in cancer cells but not in normal cells, and that the Ad-Lp-E1A vector is selectively toxic to cancer cells but not to normal cells.

The Ad-Tyr-E1A vector was directly cytotoxic *in vitro* predominantly to tumor cell lines that corresponded to the origin of the tyrosinase promoter (melanoma cells), whereas they were much less toxic to the breast and ovarian cancer cells. The Ad-Lp-E1A vector was much more toxic to breast and ovarian cancer cells than to melanoma cell lines. Thus, the L-plastin-driven E1A vector was not only much more toxic to cancer cells than to noncancer cells of the same epigenotype, but this vector also was much more toxic to breast and ovarian cancer than to other types of nonepithelial cancer.

We were also able to show that this tumor-specific cytolytic effect extended into the *in vivo* setting by injecting the vectors into the subcutaneous nodules of either breast cancer cells or melanoma cells in a SCID mouse human tumor xenograft. These experiments showed that the Ad-Lp-E1A vector could transiently suppress the growth of the MCF-7 or MDA-MB-468 breast cancer cell lines and could suppress those cells even after they had grown into an established tumor nodule. In addition, the Ad-Tyr-E1A vector transiently suppressed the *in vivo* growth of the TF-2 melanoma cell line in the SCID mouse model. Because the injection of the Ad-CMV-LacZ control replication-incompetent adenoviral vector into the subcutaneous tumor nodules in the SCID mice did not suppress the growth of the tumor nodules, the suppressive effect of the Ad-Lp-E1A and the Ad-Tyr-E1A vectors was most likely dependent on the replication competency of these two vectors, rather than to a consequence of an immunological reaction to the vector. In addition, the lytic effect of the Ad-Lp-E1A vector on cell lines *in vitro* was equivalent to the effect of the Ad-CMV-E1A on the tumor nodules in the SCID mice.

Thus, these data suggest that the Ad-Lp-E1A vector may ultimately be of value for the development of therapeutic vectors for the treatment of solid tumor malignancies. However, the suppressive effect of the vectors was not durable. It will be important to add a therapeutic transcription unit to these vectors so that their toxic action on tumor cells is more robust. A second change would be to engineer the vector such that it would bind only to tumor cells and not to normal cells. The effects of these changes are currently under study in our laboratory.

MATERIALS AND METHODS

Cells and cell culture. The following cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA): the MCF-7 and MDA-MB-468 human breast cancer cell lines and the 293 human embryonic kidney (HEK) cell line. The Ovcar-5 human ovarian cancer cell line was obtained from Thomas C. Hamilton of the Fox Chase Cancer Center (Philadelphia, PA). The TF-2 and Yusac-2 human melanoma cell lines were obtained from Ruth Halaban at Yale University (New Haven, CT). Explants of normal human mammary epithelial cells were obtained from Clonetics (Waldersvile, MD). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS).

Structure of the L-plastin and tyrosinase promoter E1A replication-competent adenoviral vectors. A DNA fragment that contains the E1 gene was generated from wild-type adenovirus 5 using the following PCR primers: 5′-ACGCGTCGACGCGAGTAGAGTTTTCTCCTCCG-3′ and 5′-AGCTTG TTTAAACTCGAGGACAGGCCTCTCAAG-3′ (GeneAmp XLPCR kit, Perkin Elmer). The 5200-bp PCR fragment was digested with *Sal*I and *Pme*I restriction enzymes and then ligated into the pShuttle plasmid [14], which had been cut with *Sal*I and *Pme*I to produce a new shuttle plasmid (pADshuttle) which contains a complete E1 gene but a deleted E1A promoter. The promoter fragments were inserted between the *Not*I and *Sal*I sites of the pADshuttle (Fig. 1). The L-plastin *Sca*I promoter was inserted into pBluescipt Sk+ after it was excised from AdLpLaz [7]. The L-plastin *Sca*I promoter fragment was then cut from the pbluescript Sk+ with *Not*I and *Xho*I. It was then ligated into the pAD shuttle plasmid. The human tyrosinase promoter-enhancer was synthesized by PCR from human Yusac-2 genome with three pairs of primers: HTP1, 5′-CCGGAATTCATTCTAACCATAAGAATTAA-3′, and HTP2, 5′-ACGCGTCGACGGAACTGGCTAATTGGAGTC-3′; TE1, 5′-ATTTGCGGCCGCAATTCTGTCTTCGAGAACAT-3′, and TE2, 5′-CGCG-GATCCATGGAAATGCTGCCTCTG-3′; HEN1, 5′-CGCGGATCCAATTCTTC-GAGAACAT-3′, and HEN2, 5′-CCGGAATTCATGGAAATGCTGCCTCTG-3′. The HTP fragment was cut with *Sal*I and *Eco*RI. The HEN fragment was cut with *Eco*RI. The vectors generated were sequenced. After sequencing, this *Not*I and *Sal*I fragment was inserted into the pAD shuttle vector. We also synthesized the CMV promoter with PCR and inserted it into the pAD shuttle plasmid. The vector has the same regulatory elements left in the E1A and E1B region, including the vector-packaging signal, as reported by Rodriguez *et al.* [4].

The replication-competent adenoviral vectors under the control of the L-plastin, tyrosinase, and CMV promoters were prepared by standard homologous recombination techniques using the pAD shuttle plasmids with AdEasy-1 (provided by Tong-Chuan He and Bert Vogelstein [14] of the Howard Hughes Medical Institute, Johns Hopkins Oncology Center) in BJ5138. After cutting with *PacI*, the adenovirus DNA plasmids, which contain different promoters, were transfected into HEK 293 cells. Each recombinant adenoviral vector was isolated from a single plaque and expanded in HEK 293 cells. Viral DNA was treated and analyzed by PCR to confirm the structure of the E1A promoters in the virus. The L-plastin promoter was sequenced to confirm its structure.

The vector for experimentation was prepared by infecting 30 15-cm tissue culture plates of HEK 293 cells and by harvesting the detached cells after 48 hours. The viral particles remained associated with the cells. Cells were collected by centrifugation at 400*g* for 5 minutes at 4°C. The cells were resuspended in 10 mL of cold PBS (free of $Ca2^{+}$ and $Mg2^{+}$), and were lysed with three cycles of freezing and thawing. The cells were collected by centrifugation at 1500*g* for 10 minutes at 4°C. The supernatant was placed on a gradient prepared with equal parts of cesium chloride in phosphate-buffered saline (PBS; 1.45 g/mL and 1.20 g/mL), and then centrifuged for 3 hours at 15,000*g* at 12°C. The virus band was removed, rebanded in a preformed cesium chloride gradient by ultracentrifugation for 18 hours, and dialyzed against cold PBS, pH 7.4, containing 10 mM $MgCl_2$ and 10% glycerol. Titers of purified adenoviral vectors were determined by spectrophotometry and by plaque assays.

Western blot analysis of adenovirus E1A protein expression. For detection of the E1A protein, 2×10^5 cells in six-well culture plates were infected with Ad-Lp-E1A and Ad-CMV-E1A at an MOI of 10. Cells were collected and lysed in cell lysis buffer (50 mM Hepes, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, 10 mM NaF, 10 ng/mL aprotinin, 10 ng/mL leupeptin, 1 mM DTT, and 1 mM sodium vanadate) 24 hours after viral infection, incubated for 60 minutes on ice, and centrifuged at 1500*g* for 10 minutes at 4°C. The supernatants were transferred to Eppendorf tubes and were kept at 100°C for 5 minutes. We added 10 μg of protein to each lane of the SDS PAGE gel. Protein was analyzed by immunoblotting with monoclonal antibody against the wild-type Ad5 E1A protein. Reactivity was visualized by enhanced chemiluminescence (Amersham Life Sciences, Inc., Arlington Heights, IL).

In vitro viral replication assay. Monolayer cell cultures in six-well dishes (1×10^5 cells/well) were infected with the Ad-Lp-E1A, Ad-Tyr-E1A, and the adenoviral vector with the CMV promoter driving the E1A gene (Ad-CMV-E1A), at an MOI of 10 pfu/cell. The virus was removed 2 hours after inoculation. The cells were then washed twice with PBS and incubated at 37°C for 72 hours. Lysates were prepared with three cycles of freezing and thawing. Serial dilutions of the lysates were titered on HEK 293 cells.

Cytopathic effect assays. Cells were passaged 24 hours before infection with adenoviral vectors at the indicated MOI. Photomicrographs were taken on days 3, 5, and 9 after infection.

In vivo gene transfer to human breast cancer xenografts and human melanoma xenograft. Subcutaneous tumor nodules were established by subcutaneous injection of 5–10 × 10^6 MDA-MB-468, MCF-7 breast cancer cells or TF-2 melanoma cells suspended in 0.1 ml of PBS into the flanks of female SCID mice aged 5–6 weeks. Tumor nodules were allowed to grow subcutaneously to approximately 6–7 mm in diameter. For intratumoral injection of the viral vectors, 50 μL of viral particles (10^8 pfu) suspended in PBS were injected using a 25-gauge needle. Tumor size was measured at the indicated times after injection in their longest dimension and the dimension at 90° to that measurement. Tumor volumes were calculated using the following formula: (length × $width^2$)/2. Tumor volumes were normalized to 100% on day 0 (V/V_0). Results are expressed as the fractional tumor volume (mean ± SD) at each time point compared with that on the day of injection.

ACKNOWLEDGMENTS

We recognize support to A.D. from the United States Anny (DAMD 17-99-9457), The Audrey Demas Melanoma Research Fund, The Anthony Dewitt Frost Memorial Fund for Melanoma Research, The Greenwich Breast Cancer Collective, The Hull Development Fund, The Breast Cancer Research Foundation, The Susan Komen Foundation, and the George and Barbara Bush Leukemia Research Fund.

RECEIVED FOR PUBLICATION APRIL 12, 2001; ACCEPTED JULY 22, 2002.

REFERENCES

1. Haj-Ahmad, Y., and Graham, F. (1986). Characterization of an adenovirus type 5 mutant carrying embedded inverted terminal repeats. *Virology* **153**: 22–34.
2. Bett, A., Haddara, W., Prevec, L., and Graham, F. (1994). An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. USA* **91**: 8802–8806.
3. Pang, S. (2000). Targeting and eradicating cancer cells by a prostate-specific vector carrying the diphtheria toxin A gene. *Cancer Gene Ther.* **7**: 991–996.
4. Rodriguez, R., *et al.* (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res.* **57**: 2559–2563.
5. Miyatake, S. I., *et al.* (1999). Hepatoma-specific antitumor activity of an albumin enhancer/promoter regulated herpes simplex virus *in vivo*. *Gene Ther.* **6**: 564–572.
6. Heise, C., and Kirn, D. H. (2000). Replication-selective adenoviruses as oncolytic agents. *J. Clin. Invest.* **105**: 847–851.
7. Chung, I., Schwartz, P. E., Crystal, R. G., Leavitt, J., and Deisseroth, A. B. (1999). Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cells. *Cancer Gene Ther.* **2**: 99–106.
8. Lin, C. S., *et al.* (1997). The murine L-plastin gene promoter: identification and comparison with the human L-plastin gene promoter. *DNA Cell Biol.* **16**: 9–16.
9. Lin, C. S., *et al.* (1993). Characterization of the human L-plastin gene promoter in normal and neoplastic cells. *J. Biol. Chem.* **268**: 2793–2801.
10. Lin, C. S., *et al.* (1993). Human plastin genes. Comparative gene structure, chromosome location, and differential expression in normal and neoplastic cells. *J. Biol. Chem.* **268**: 2781–2792.
11. Siders, W. M., *et al.* (1998). Melanoma-specific cytotoxicity induced by a tyrosinase promoter-enhancer/herpes simplex virus thymidine kinase adenovirus. *Cancer Gene Ther.* **5**: 281–291.
12. Siders, W. M., *et al.* (1998). T cell- and NK cell-independent inhibition of hepatic metastases by systemic administration of an IL-12-expressing recombinant adenovirus. *J. Immunol.* **160**: 5465–5474.
13. Siders, W. M., *et al.* (1996). Transcriptional targeting of recombinant adenoviruses to human and murine melanoma cells. *Cancer Res.* **56**: 5638–5646.
14. He, T. C., *et al.* (1998). A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* **95**: 2509–2514.
15. Peng, X. Y., Rutherford, T., Fujii, T., Schwartz, P., and Deisseroth, A. (2001). The use of the L-plastin promoter for adenoviral mediated tumor-specific gene expression in ovarian and bladder cancer cell lines. *Cancer Res.* **61**: 4405–4413.
16. Ohashi, M., *et al.* (2001). Target gene therapy for α-fetoprotein-producing hepatocellular carcinoma by E1B55k-attenuated adenovirus. *Biochem. Biophys. Res. Commun.* **282**: 529–535.

Cancer Gene Therapy (2003) 10, 388–395

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Cytotoxic effect of replication-competent adenoviral vectors carrying L-plastin promoter regulated E1A and cytosine deaminase genes in cancers of the breast, ovary and colon

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Prodrug activating transcription unit gene therapy is one of several promising approaches to cancer gene therapy. Combining that approach with conditionally replication-competent viral vectors that are truly tumor specific has been an important objective of recent work. In this study, we report the construction of a new conditionally replication-competent bicistronic adenoviral vector in which the cytosine deaminase (CD) gene and the E1a gene are driven by the L-plastin tumor-specific promoter (AdLpCDIRESE1a). A similar vector driven by the CMV promoter has also been constructed (AdCMVCDIRESE1a) as a control. We have carried out *in vitro* cytotoxicity in carcinomas of the breast, ovary and colon, and *in vivo* efficacy studies with these vectors in an animal model of colon cancer. While the addition of the AdLpCDIRESE1a vector to established cancer cell lines showed significant cytotoxicity in tumor cells derived from carcinomas of the breast (MCF-7), colon (HTB-38) and ovary (Ovcar 5), no significant toxicity was seen in explant cultures of normal human mammary epithelial cells (HMEC) exposed to this vector. The addition of 5-fluorocytosine (5FC) significantly increased the cytotoxicity in an additive fashion of both the AdLpCDIRESE1a and AdCMVCDIRESE1a vectors as well as that of the AdLpCD replication incompetent vector to established tumor cell lines. However, no significant cytotoxicity was observed with the addition of 5FC to explant cultures of normal human mammary epithelial cells that had been exposed to the L-plastin-driven vectors. Studies with mixtures of infected and uninfected tumor cell lines showed that the established cancer cell lines infected with the AdLpCDIRESE1a vector generated significant toxicity to surrounding uninfected cells (the "bystander effect") even at a ratio of 0.25 of infected cells to infected + uninfected cells in the presence of 5FC. The injection of the AdLpCDIRESE1a vector into subcutaneous deposits of human tumor nodules in the nude mice was potentiated by administering 5-FC by intraperitoneal injection. This treatment resulted in a decreased tumor size and a decreased tumor cell growth rate. The mice treated with a combination of the AdLpCDIRESE1a vector intratumoral injection and intraperitoneal 5FC injections lived much longer than the other experimental groups exposed to the viral vector alone or to the combination of the intratumoral AdLpCD replication incompetent vector injections plus intraperitoneal 5-FC injections. These encouraging results with our newly constructed AdLpCDIRESE1a vector suggest a need for further study of its utility in a preclinical model of intracavitary therapy of pleural or peritoneal carcinomatosis.
Cancer Gene Therapy (2003) **10,** 388–395. doi:10.1038/sj.cgt.7700579

***Keywords:* adenoviral vectors; cancers of the breast; ovary and colon; L-plastin; E1A; cytosine deaminase/5-fluorocytosine**

Although only a small number of cancer gene therapy trials have been completed, and fewer still have been successful, gene therapy is still regarded as one of the most exciting new directions in cancer treatment. Many of these trials have used adenoviral vectors to carry prodrug activation transcription units to sensitize the cancer cells to chemotherapeutic agents and to reduce side effects of therapy on the normal tissues of the body. Adenoviral vectors carrying the *Escherichia coli* or yeast cytosine deaminase (CD) gene,[1] which catalyzes the conversion of the relatively harmless drug, 5-fluorocytosine (5FC) into the cytotoxic agent, 5-fluorouracil (5FU), has been one of the most frequently used approaches because the levels of 5FU that can be delivered with this system can kill both dividing as well as nondividing cells, and because of the incorporation of very high levels of 5FU into RNA as well as DNA.[2,3] This results in the death of the nondividing cells because of the interruption of protein synthesis.

Adenoviral vectors are among the most widely used vector systems for the introduction of genetic elements into somatic cells for cancer gene therapy. The major limitation of the adenoviral vectors is the stimulation of a vigorous immune response. In cancer gene therapy, this may not always be a disadvantage. Adenoviral vectors have some advantages which other vector systems do not

Received April 22, 2002.
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share: transgene expression in nondividing cells, and the broad range of cells which they infect.

The ability to infect normal host cells as well as cancer cells, and to express the therapeutic transgene in dividing as well as nondividing cells, is a major cause of toxicity as well as efficacy.[4,5] The use of tumor or tissue-specific transcriptional promoters is one of the current approaches to solving the problem of toxicity to normal cells.[6,7] Accordingly, our laboratory has constructed adenoviral vectors using the L-plastin promoter, which is a tumor-specific promoter, which drives the expression of therapeutic transgenes in tumor cells but not in normal cells.[8–10] Experiments carried out by Xue Yuan Peng of our laboratory have recently shown a tumor-specific suppressive effect of the L-plastin-driven CD transcription unit carried by replication-deficient adenoviral vectors to breast, ovarian and bladder cancers.[9]

In the experiments summarized in this report, our goal was to study the use of a replication competent adenoviral vector in which the L-plastin tumor-specific promoter was driving the expression of both the CD and E1a genes in established human tumor cell lines derived from carcinomas of the breast, ovary and colon, and in an animal model of human carcinoma of the colon.

Materials and methods

Human cells lines derived from carcinomas of the breast, ovary and colon

The human breast cancer cell line (MCF-7), the transformed human kidney cell line (HEK293) and the human colon cancer cell line (HTB-38) were purchased from American Type Culture Collection (ATCC). The human epithelial ovarian cancer cell line (Ovcar-5) was obtained from Dr Thomas C Hamilton of Fox Chase Cancer Center, Philadelphia, PA. The human mammary epithelial cell line (HMEC) was purchased from Bio-Whittaker, Walkersville, MD. The MCF-7, Ovcar-5 and HEK293 cell lines were grown in Dulbecco's Eagle's medium supplemented with 10% heat-inactivated newborn calf serum (NBC) and HTB-38 cells were grown in McCoy's 5A modified medium supplemented with 10% NBC. The HMEC cell line was grown in a growth medium supplemented with hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), epidermal growth factor (10 ng/ml) and bovine pituitary extract (26 μg/ml). All the cell lines were maintained in a 5% CO_2, humidified tissue culture incubator at 37°C. The HTB-38 cell line was tested for mouse hepatitis virus before inoculating the mice.

Construction of adenoviral vectors carrying a bicistronic therapeutic transcription unit regulated by the L-plastin tumor-specific promoter

Wild-type adenovirus type 5 (Ad5WT) was obtained from the ATCC. A replication-deficient adenoviral vector carrying the L-plastin-driven CD gene (AdLpCD) was previously engineered in our laboratory.[8,9] In this current paper, a new replication-competent bicistronic adenoviral vector carrying the L-plastin-driven CD and E1a genes in a single continuous bi-cistronic transcription unit was constructed. The CD gene was synthesized by PCR from the pShuttleLp-CD plasmid using the primers XhoI (ccgctcgagaggctaatgtcgaat) and XbaI (gctctagattaccgtttgtaatcgat). The intraribosomal entry site (IRES) sequence, obtained by PCR from pCITE (2+) with the primers *Spe*I (ggactagtggttattttccaccatattgccgt) and *Sal*I (acgcgtcgacggtattatcatcgtgtttttca) was inserted between the *Xba*I/*Spe*I and *Sal*I restriction sites. A 2.4-kb fragment of the L-plastin promoter, truncated by Injae Chung of our laboratory from the 5-kb promoter extending from the nucleotide −2265 of the 5′ region of the L-plastin promoter to +18 bp from the transcription initiation site of the L-plastin gene,[8] was inserted between *Not*I and *Xho*I of pShuttle. This vector is designated AdLpCDIRESE1a. After linearizing the constructed plasmid with *Pme*I, it was cotransformed into the *E. coli* strain BJ5183 with pAdEasy-1 viral DNA plasmid for homologous recombination. Another bicistronic virus with the CMV tumor nonspecific promoter, instead of the L-plastin promoter, was also constructed. This vector is designated AdCMVCDIRESE1a. The maps of the newly constructed replication competent viruses are shown in Figure 1. Following the validation of the structure of the recombinant AdLpCDIRESE1a and ACMVCDIRESE1a vectors by restriction endonuclease digestion analysis, the vectors were transfected into HEK293 cells where the E1 gene is complemented *in vivo*.[11]

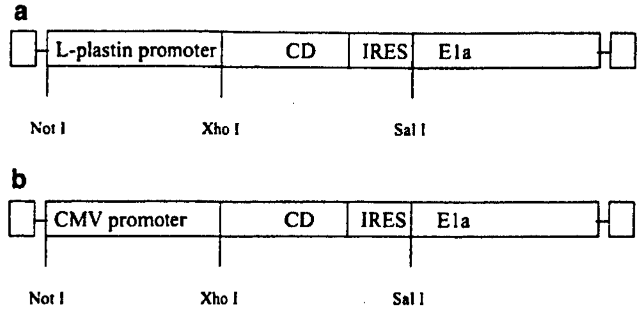

Figure 1 The map of newly constructed conditionally replication-competent adenoviral vectors. E1a is transcriptionally coupled to the cytosine deaminase (CD) gene by an IRES in both vectors. In the AdLpCDIRESE1a vector (**a**), the bicistronic transcription unit is driven by the L-plastin promoter. In AdCMVCDIRESE1a vector (**b**), the same transcription unit is driven by the CMV promoter.

The number of infectious adenoviral particles in the stocks of both AdLpCD and AdLpCDIRESE1a, expressed as plaque-forming units (PFU), was determined by limiting dilution assay of plaque formation in HEK293 cells as described previously.[12]

Analysis of the expression of the CD gene in the AdLpCDIRESE1a vector

The expression of the CD gene in the bicistronic transcription units of the AdLpCDIRESE1a vector was measured by extracting RNA from cells infected with the vector and then the RT-PCR of this mRNA was used to generate cDNA for molecular weight analysis as described previously.[13] The primers for this reaction were: *Xho*I (ccgctcgagaggctaatgtcgaat) and *Xba*I (gctctagattaccgtttgtaatcgat), which come from the CD gene. Briefly, the first strand DNA was synthesized by using "Superscript-II reverse transcriptase" enzyme at 25°C for 10 minutes. Following the termination of the reaction at 95°C for 5 minutes, the tubes were incubated with RNase H at 37°C for 20 minutes. Then, the product of the first strand cDNA was amplified by using Ready to go PCR-beaded tubes (Amersham Pharmacia Biotech) for 30 cycles of each which included a denaturation period of 30 seconds at 94°C and annealing period of 60 seconds at 54°C. The predicted molecular weight of the CD fragment generated by this PCR reaction is around 1.2 kb.

Cytopathic effect of the vectors

The HTB-38, MCF-7, Ovcar-5 human tumor cell lines and the normal human mammary epithelial cell cultures were seeded at a density of 200,000 cells/well in six-well plates. After 24 hours, the cells were infected at various MOI by exposure to the following vectors: Ad5WT, AdLpCD and AdLpCDIRESE1a and AdCMVCDIRESE1a. Following the culture of the infected cells for an additional 4 days, the cells were examined for cytopathic effect (CPE) by light microscopy.

Functional analysis of the CD gene in the adenoviral vector backbone

The HTB-38, MCF-7, Ovcar-5 and HMEC cell lines were seeded at a density of 200,000 cells/well in six-well plates. The cells were then exposed to the AdLpCD and AdLpCDIRESE1a vectors at the following MOI (0, 0.1, 1, 10, 30, 80). After 24 hours, the cultures were supplemented with sufficient 5-FC, purchased from Sigma Chemical Co., to make the cultures 500 μM with respect to 5FC. Following the incubation of the cells for an additional 4 days, the cells were trypsinized and the surviving cells counted by the trypan blue exclusion test as described previously.[14] The percentage of surviving infected cells was calculated by taking the ratio of the surviving cells to the total cells, which were incubated without vector being added to the cultures.

Bystander effect assay

Following exposure of the cell lines to the vectors under conditions that would lead to infection of 100% of the cells (MOI of 80 with AdLpCD and AdLpCDIRESE1a vectors), the infected cell lines HTB-38, MCF-7, Ovcar-5 and HMEC were mixed individually with the corresponding uninfected cells at the following ratios of infected to uninfected cells: 0, 0.05, 0.25, 0.50 and 1.0. The seeding density of the total number of cells was 2×10^5 cells/well into six-well plates. After 24 hours, sufficient 5-FC was added to the cultures to generate a 5FC concentration of 500 μM. Following the incubation of the cells for an additional 4 days, the cells were trypsinized and the surviving cells counted with the trypan blue exclusion test as described previously.[14]

Animal studies

The HTB-38 cells (3×10^6) suspended in PBS were injected s.c. in Balb/C nude/nude mice. The mice were purchased from Charles River Laboratories, Inc., Wilmington, DL. After sufficient time had elapsed to allow for the development of the tumor nodule to a size in the 50 mm^3 range, the mice were randomly divided into five groups and 10^8 PFU of the AdLpCDIRESE1a or AdLpCD vectors were injected intratumorally into seven mice for each of the treatment groups, and with phosphate-buffered saline (PBS) for the control group. Then, 500 mg/kg of 5FC was injected once a day for 7 days intraperitoneally into experimental groups of animals in which the sensitization of the infected tumor cells to 5FC by CD was to be studied, and with equal amounts of PBS into animals in which the growth of the tumor nodule in the absence of treatment was to be studied. Then, tumor sizes were measured every 6 days. Tumor volumes were calculated by the formula "volume = length × (width^2/2)".[9]

Statistical analysis

Results of the *in vitro* cytotoxicity tests were evaluated by a nonlinear regression method and the maximum cytotoxicity of each of the viruses was compared by the Student's *t*-test. One-way ANOVA (with LSD *post hoc* comparisons) and Mann–Whitney tests were used for the comparison of tumor volumes. Tumor growth rates were evaluated by regression analysis. Survival analyses were performed according to Kaplan–Meier method and the log-rank test was used for survival comparisons.

Results

Expression of the CD gene in vector-exposed tumor cells derived from carcinomas of the breast, ovary, and colon

The MCF-7 human breast cancer cell line, the Ovcar-5 human ovarian cancer cell line, and the HTB-38 human colon cancer cell line were seeded at a density of 200,000 cells/well in six-well plates. At 24 hours, these cells were exposed to the Ad5WT, AdLpCD, AdLpCDIRESE1a and AdCMVCDIRESE1a vectors. Following a period of incubation of between 12 and 16 hours, the cells were trypsinized and washed with PBS. Total RNA was then isolated from these cells, and cDNA was then generated using the primers specific for the CD coding transcripts. Portions of the CD gene were synthesized and amplified from the mRNA of the gene by RT-PCR. The bands produced by PCR from the CD cDNA of MCF-7 cells are shown in Figure 2. All the cell lines used in the current study yielded similar results. The species shown in Figure 2, which were obtained from the amplification of the RNA from the CD vector cells show that the expected molecular weight for CD is around 1.2 kb. In contrast, the expected CD fragments were not seen in the RNA from the cells infected by the control vector. These data show that the AdLpCD, AdLpCDIRESE1a and AdCMVCDEIRESE1a vectors were expressing CD coding

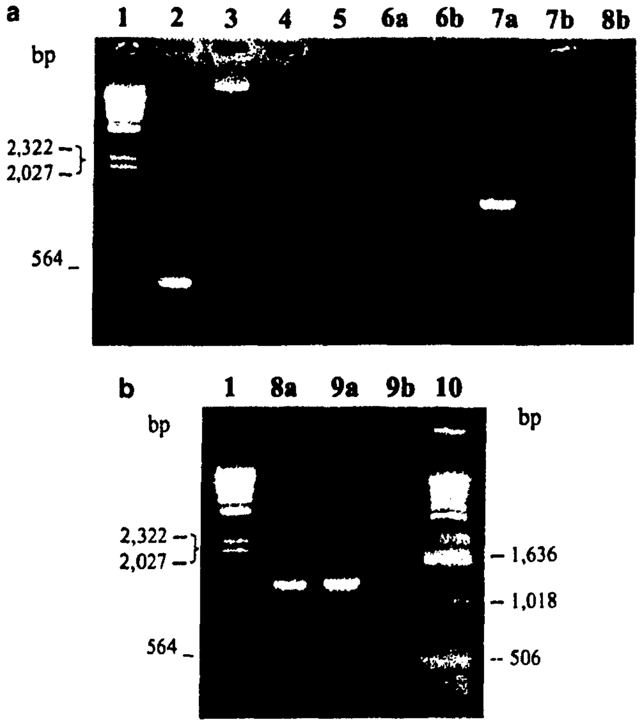

Figure 2 Expression of CD gene in MCF-7 cells. Following a period of incubation of between 12 and 16 hours, the cells infected with AdLpCD, AdLpCDIRESE1a and AdCMVCDIRESE1a were trypsinized and washed with PBS. cDNA was then generated using the primers specific for the CD coding transcripts from the isolated total RNA of infected MCF-7 cells. Portions of the CD gene were synthesized and amplified from the mRNA of the gene by RT-PCR. The DNA synthesized from the vectors, the plasmid and control RNA along with DNA markers were analyzed by electrophoresis through an 1% agarose gel and ethidium bromide staining. Lane 1, λ DNA *Hin*dIII fragments; lane 2, control RNA; lane 3, AdLpCDIRES plasmid; lane 4, wild-type adenovirus; lane 5, no virus infection; lane 6, AdLpCD; lane 7, AdLpCDIRESE1a (12 hour infection); lane 8, AdLpCDIRESe1a (16 hour infection); lane 9, AdCMVCDIRESE1a; lane 10, 1 kb DNA marker (**a**: with reverse transcriptase (RT), **b**: without RT). All of the recombinant vectors carrying the CD gene yielded a visible CD band, which is 1.2 kb with RT.

mRNA sequences. Our previous results showed that the ratio of the β-galactosidase levels in various tumor cell lines exposed to AdCMVLacZ and AdLpLacZ was around 2, however, it was 9 for CCD, which is a minimum deviation cell line.[9] MCF-7 cells have been previously reported to express L-plastin by Chung et al of our laboratory.[8]

Analysis of CPEs generated in normal mammary epithelial cells and in cell lines exposed to newly constructed vectors

The CPEs generated by exposure to the Ad5WT, AdCMVCDIRESE1a, AdLpCDIRESE1a and AdLpCD vectors were studied in the following tumor cell lines: MCF-7, Ovcar-5 and HTB-38, and in addition, in explants of normal HMEC at varying MOI. The replication-competent vectors (AdWT, AdLpCMVCDIRESE1a and AdLpCDIRESE1a) produced a strong CPE following 4 days of infection in tumor cells (data not shown). The wild-type adenovirus (AdWT) and the CMV-driven bicistronic vectors (AdCMVCDIRESE1a) produced a strong CPE on HMEC, whereas the L-plastin-driven vectors: both the replication-competent AdLpCDIRESE1a vector at 80 MOI, and the replication-incompetent AdLpCD vector at 80 MOI generated little or no CPE in HMEC (data not shown). In a very recent study, we have reported that adenoviral vectors carrying L-plastin-driven E1a caused significant CPE in various tumor cell lines but not in HMEC line.[10] These studies[8–10] showed that the L-plastin promoter was tumor specific in promoting the CD and E1a gene expression in tumor cell lines derived from carcinomas of the breast, ovary and colon and in the generation of a cytopathic effect.

Analysis of cytotoxicity generated in vitro *by vectors at different MOI in tumor cell lines derived from carcinomas of the breast, ovary and colon*

In this study, *in vitro* cytotoxicity tests were carried out with both replication-competent and -incompetent L-plastin-driven vectors. As shown in Figure 3, exposure of human tumor cell lines to the replication competent AdLpCDIRESE1a vector generated a significant cytotoxic effect starting at an MOI of 1. The cytotoxic effect was characterized by rounding up and lifting off of the cell monolayer. The maximum predicted cytotoxic effect of the replication-competent AdLpCDIRESE1a vector (without 5FC treatment) was seen at an MOI of 80. The percentage of cells killed in the MCF-7 human breast cancer cell line and in the HTB-38 human colon cancer cell line at a MOI of 80 (70.9 and 66.1%) was greater than that seen in the Ovcar-5 cell line (46.5%). This may be because of the lower percentage of cells which are positive for the receptor needed for infection by the adenoviral vector in the Ovcar-5 cell line as compared with the other cell lines.[9]

In contrast, the maximum cytotoxic effect of the AdLpCD replication-incompetent vector at an MOI of 80 without 5FC treatment was 19.3% in MCF-7, 21.6% in HTB-38 and 16.1% in Ovcar-5 cells (see Fig 3). The cytotoxic effect of the replication-competent vector was significantly higher than that of the replication-incompetent vector in all studied tumor cell lines ($P<.0001$). In contrast to the experience with the tumor cell lines, the percentage of the HMEC cells exposed to vectors at an MOI of 80 which were dead was less than 20% after 5 days of the infection for both replication-competent and replication-incompetent vectors when they were driven by the L-plastin promoter.

The maximum predicted cytotoxicity after addition of 5-FC to the AdLpCDIRESE1a vector infected cells was 91.3% in MCF-7, 94.9% in HTB-38 and 87.3% in Ovcar-5 cells. Thus, addition of 5-FC following exposure of tumor cell lines to the replication-competent vector AdLpCDIRESE1a significantly increased the cytotoxicity in all tumor cells ($P<.001$, $<.01$ and $<.0001$, respectively). The addition of 5-FC, also caused significantly more cell death in the MCF-7 breast cancer cell line, in the

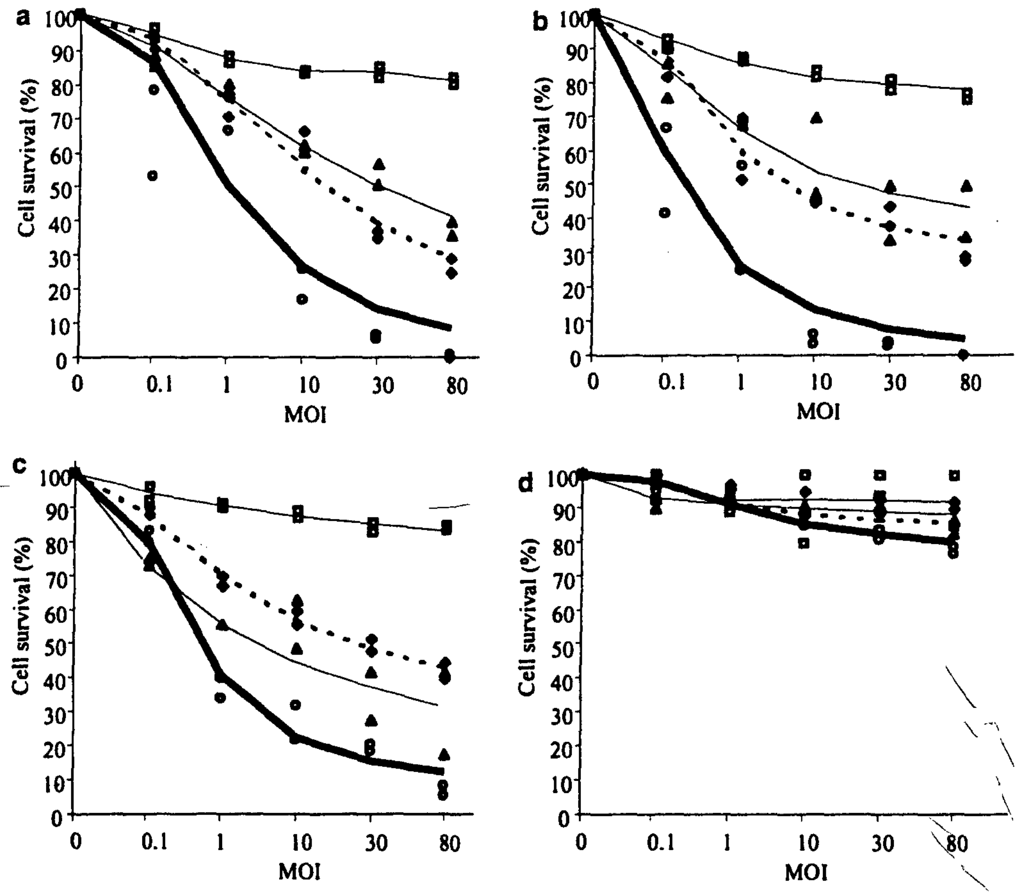

Figure 3 Results of an *in vitro* cytotoxicity test. The tumor cell lines (**a**: MCF-7 cells; **b**: HTB-38 cells; **c**: Ovcar-5 cells), and explants of normal human mammary epithelial cells (**d**) were seeded in duplicate at a density of 200,000 cells/well in a six-well plate. They were then infected at 0, 0.1, 1, 10, 30, 80 MOI with AdLpCDIRESE1a (--- ◆ ---) and AdLpCD (–■–). Another set of the above experiments were set up in duplicate and following infection with the same vectors were incubated at 500 μM 5FC for 4 days (AdLpCDIRESE1a+5FC (–○–); AdLpCD+5FC (–▲–). The percentage of surviving cells was counted by trypan blue exclusion (markers represent observed data and lines represent the predicted result).

HTB-38 colon cancer cell line, and in the Ovcar-5 ovarian cancer cell line infected with replicating-incompetent vector when compared to vector alone (58.6% $P<.0001$, 57.0% $P<.01$ and 67.7% $P<.001$, respectively). The cytolytic effect of replication-competent vector alone appears to be additive when combined with 5-FC (see Fig 3). The change in the maximum predicted cytotoxicity of the vector in HMEC cell line after addition of 5-FC was not significant for both replication-competent vector: 13.8 *versus* 19.2%, $P>.05$ and for replication incompetent vector: 7.4 *versus* 11.1%, $P>.05$. These data show that the AdLpCDIRESE1a vector is tumor specific in its CPE and that the addition of 5FC to the cells exposed to the replication-competent vectors increased the CPE. In addition, the two therapeutic transcription units (CD and E1a) together appear to be additive in the effect of vectors on tumor cells.

Analysis of the effect of infected cells on uninfected cells (the "bystander effect")

To evaluate the effect of 5-FU generated in and released from the CD vector-infected cells on the uninfected cells, we generated mixtures of the cells infected with the AdLpCDIRESE1a and AdLpCD vectors under conditions that led to infection of 100% of the cells, with uninfected cells at the following ratios of infected to the uninfected + infected cells: 0, 0.05, 0.1, 0.25, 0.50 and 1.0. The mixtures were plated in six-well plates and then exposed to 5-FC at 500 μM. The effect of the 5-FU released from the infected cells on the vector noninfected cells was seen easily at a ratio of infected to noninfected + infected cells of 0.25 (see Fig 4). More than half of the cells were dead even when the ratio of infected cells was less than 25%. The significant by-stander effect augments the direct cytolytic effect of the bicistronic replication competent vector. In addition, the replication-competent AdLpCDIRESE1a vector with the L-plastin bicistronic transcription unit was more toxic than the replication-incompetent AdLpCD vector. Thus, the addition of the E1a transcription unit to that of the CD vectors appeared to increase the cytotoxic effect of the vector on tumor cell lines derived from carcinomas of the breast, ovary and colon. Finally, all of the effects of the vectors appeared to be tumor specific when the L-plastin promoter was used to drive the therapeutic transcription units, on the basis of

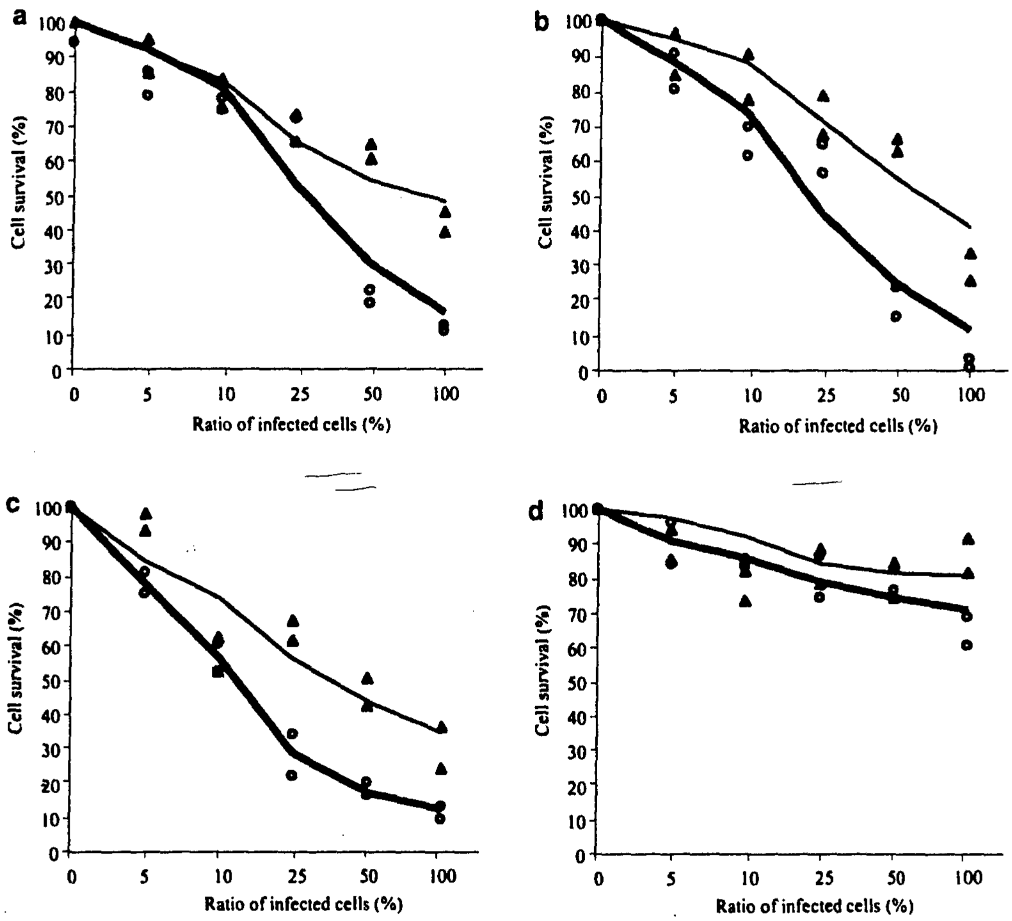

Figure 4 Results of by-stander effect assay. The MCF-7 (**a**), the HTB-38 (**b**), the Ovcar-5 (**c**) and the HMEC (**d**) cell lines were infected at an MOI of 80 with the AdLpCDRESE1a and AdLpCD vectors. The infected cells were mixed with uninfected ones in varying ratios of 0, 5, 10, 25, 50 and 100%. Then to test the cytotoxicity of released 5-FU from the infected cells, the mixture of cells were seeded in duplicate in six-well plates and incubated in 500 μM 5FC for 4 days. The percentage of surviving cells with AdLpCDIRESE1a+5FC (–△–); AdLpCD+5FC (–O–) plates was counted by trypan blue exclusion (markers represent the observed data and the lines represent the predicted results).

the comparison of the effect on normal mammary epithelial cells as compared to the effect on established human breast cancer cell lines.

Efficacy of the AdLpCDIRESE1a replication-competent vector in an in vivo *model*

The efficacy of replication-competent and replication-incompetent vector/5-FC systems was tested in nude mice bearing subcutaneous tumor nodules of the HTB-38 human colonic cancer cell line. On the 8th day following injection of HTB-38 cells, the tumor volumes were measured and the mice were randomly divided into five groups: (1) the mice injected intratumorally with the AdLpCDIRESE1a vector and then treated intraperitoneally with 5-FC; (2) the mice injected intratumorally with the AdLpCDIRESE1a vector without 5FC treatment; (3) the mice injected intratumorally with the AdLpCD vector followed by intraperitoneal 5-FC treatment; (4) the mice injected intratumorally with the AdLpCD vector without 5FC treatment and (5) control mice injected intratumorally with PBS. The average volume of tumor nodules in the control and AdLpCDIRESE1a treated groups on the first day of treatment (day 0) was lower than in the other groups (see Fig 5). The mice in all the groups were followed until the animals were killed because of the large tumor volume on the 44th day of observation following injection of the vector. Most of the mice, which were alive at the end of the 44 day post vector injection observation period, were killed because of the large size of the tumor nodules.

There were four partial remissions (4/7) in the AdLpCDIRESE1a + 5FC-treated group at the end of the first week of the treatment. The average tumor volumes of this group significantly decreased during the first week ($P = .046$). However, the tumor nodules of the responding mice started to grow after 2 weeks of drug administration. Nevertheless, the tumor growth rate in this group was significantly slower than that of the other groups during the whole study period ($P < .05$). As shown, in Figure 5, the tumor volume of the AdLpCDRESE1a + 5FC treated group was significantly lower than that of the AdLpCDIRESE1a, AdLpCD + 5-FC and control groups during the observation period. There was no significant tumor response in the AdLpCD + 5-FC group. Tumor volumes of this latter group also did not

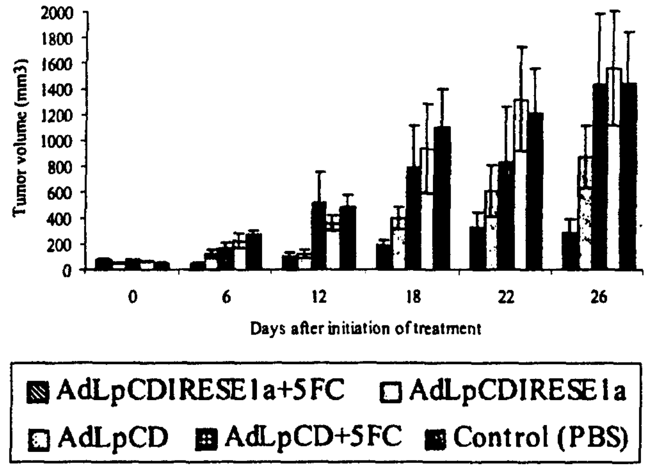

Figure 5 Effect of intratumoral injection of tumor nodules with adenoviral vectors on tumor growth. The HTB-38 cells (3 × 10[6]) were injected s.c. into nude/nude mice. Following the growth of tumor nodules to around 50 mm[3], 10[8] PFU of the AdLpCDIRESE1a and AdLpCD vectors were injected intratumorally into the mice for each of the treatment groups, and PBS for the control group. Then, 500 mg/kg of 5FC was injected intraperitoneally into experimental groups of animals and control groups and equal amounts of PBS to the vector alone groups, once a day, for 7 days. Then, tumor sizes were measured every 6 days.

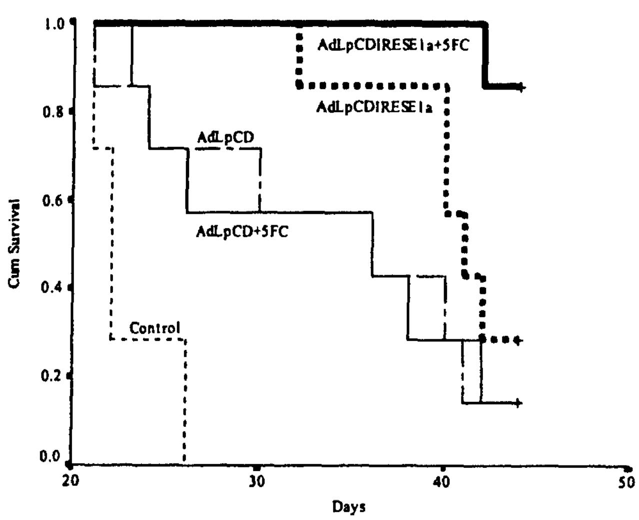

Figure 6 Kaplan–Meier survival curves of nude mice with tumor nodules from HTB-38 colon cancer. The mice in all groups were followed until death or killing because of the large tumor size or at the end of 44 days of observation. At that time, most of the mice that were alive were killed because of the large size of the tumor nodules.

significantly differ from the AdLpCD and control groups (see Fig 5).

The mice in the AdLpCDIRESE1a + 5-FC group lived significantly longer than the other groups ($P<.02$) as shown in Figure 6. While the median survival in this group was not reached during the observation period, it was 41, 36, 36 and 22 days for AdLpCDIRESE1a, AdLpCD + 5-FC, AdLpCD and control groups. All of the vector-injected groups lived longer than the mice in the control group ($P<.01$).

These data showed that the effect of the vector carrying the transcription units (E1a and cytosine deaminase) was greater in the presence of the prodrug 5-FC than the vector in the absence of 5-FC. In the latter condition, the vector is equivalent to the AdLpE1a vector. Thus, the bicistronic vector is an improvement over either vector with a single transcriptional unit (AdLpE1a or AdLpCD).

Discussion

In this report, we present the analysis of replication-competent adenoviral vectors designed to use prodrug activation transcription units driven by a tumor-specific promoter (L-plastin). The cytotoxic effect of the vector carrying both the E1A and the CD transgenes was greater than the vector which contains only the CD gene prodrug activation transcription unit without the E1a gene, both in an *in vitro* cell line experiment as well as in an *in vivo* experiment in human tumor xenograft models.

Prodrug activation transcription unit gene therapy is one of many new and promising approaches to cancer treatment. Previous reports have shown the tumor suppressive effect of adenoviral vectors carrying the CD gene/5-FC system on various tumor cell lines and *in vivo* models.[2,9,15] However, most of these reports are focused on replication-incompetent vector systems. The infectivity of normal as well as tumor cells by the adenoviral vector has represented a disadvantage for adenoviral vectors, since the expression of the therapeutic transgenes in the normal cells generates toxic side effects. In order to overcome this limitation, many groups have been focusing on tumor- or tissue-specific gene promoters to reduce side effects. Our laboratory has recently reported experiments involving vectors carrying the tumor-specific L-plastin-driven genes.[8,9] These results showed that the replication-competent viral vector can kill the tumor cell directly by the oncolytic effect of the virus.

The goal of the replication-competent viral vectors is the direct killing of the target tumor cell by the oncolytic effect of the virus.[16] The use of the L-plastin tumor-specific promoter to drive the expression of the E1a gene as well as the prodrug activation transcription unit has been shown in our work to increase the therapeutic effect over that seen with replication-competent vectors in an additive fashion without increasing toxicity.[10]

It is worth nothing that both replication-competent and -incompetent vectors, which carried CD/E1a or CD transcription units under the control of the L-plastin tumor-specific promoter caused no significant CPE on HMEC. However, the wild-type adenovirus and similarly constructed replication-competent CMV-driven adenoviral vectors caused significant CPE to the HMEC by the 4th day after an initial exposure to the vector. These data show that the L-plastin-driven CD or CD/E1a vectors are selectively toxic to the tumor cell lines without being toxic to the normal cells.

The conditionally replication-competent CD vectors killed almost all of the MCF-7 human breast cancer cells and the HTB-38 human colon cancer cells in the presence of 5FC. In both replication-competent and replication-incompetent virus/5-FC systems, active drug released from the infected cells may cause significant cell death among the uninfected cells. This significant *in vitro* by-stander effect seems to increase the efficacy of this virus/prodrug therapeutic system.

We tested the *in vivo* effect of the AdLpCDIRESE1a viral vector by injecting the vectors intratumorally into subcutaneous nodules of the human colonic cancer cell line HTB-38 followed by intraperitoneal injections (twice a day) of 5FC in quantities sufficient to generate a peak concentration of 500 μM. The injection of the Ad-LpCDIRESE1a vector in the absence of 5FC did not cause tumor response, but decreased the tumor growth rate. Addition of 5FC yielded approximately 60% objective tumor response rate along with a decrease in tumor growth rate. The mice in this group treated with the AdLpCDIRESE1a/5FC system lived significantly longer than the mice in other groups.

The use of the L-plastin promoter to drive the CD gene and the E1a gene produce a vector that is at the same time replication competent in tumor cells and which can sensitize these cells to the effects of chemotherapy. These vectors might be useful in intracavitary therapy, where the dose of chemotherapy drugs is usually limited by the local toxicity of the chemotherapy on the normal peritoneal cells. With these vectors, one can selectively increase the doses of 5FC inside the tumor cells. These vectors are currently being engineered so that they will not bind to the normal peritoneal epithelial surface. Once that process is completed, they may be tested in preclinical animal models to determine if the vectors are able to suppress spontaneously occurring tumors like ovarian carcinomatosis

Acknowledgments

The authors acknowledge support from the George and Barbara Bush Leukemia Research Fund, the Anthony Dewill Frost Melanoma Research Fund, the Audrey Demas Fund, the Breast Cancer Research Foundation, the United States Army Breast Cancer Research Program (DAMD 17-99-1-9457), and a laboratory supply fund from the Sidney Kimmel Cancer Center. Hakan Akbulut recognizes a fellowship from the National Cancer Institute of the United States of America and from the TUBITAK of the Republic of Turkey.

References

1. Lal S, Lauer UM, Niethammer D, et al. Suicide genes: past, present and future perspectives. *Immunol Today*. 2000;21:48–54.
2. Hirschowitz EA, Ohwada A, Pascal WR, et al. *In vivo* adenovirus-mediated gene transfer of the *Escherichia coli* cytosine deaminase to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. *Hum Gene Ther*. 1995;6:1055–1063.
3. Bentires-Alj M, Hellin A-C, Lechantewur C, et al. Cytosine deaminase suicide gene therapy for peritoneal carcinomatosis. *Cancer Gene Ther*. 2000;7:20–26.
4. Shenk TE. Adenoviridae: the viruses and their replication. In: Knipe DM, Howley PM eds. *Field's Virology*, 4th edn. Philadelphia: Lippincott Williams & Wilkins; 2001:2265–2300.
5. Joss K, Turka LA, Wilson JM. Blunting of immune responses to adenoviral vectors in mouse liver and lung with CTLA4Ig. *Gene Ther*. 1998;5:309–319.
6. Nettelbeck DM, Jerome V, Muller R. Gene therapy: designer promoters for tumor targeting. *Trends Genet*. 2000;16:174–181.
7. Pang S, Taneja S, Dardashti K, et al. Prostate tissue specificity of the prostate-specific antigen promoter isolated from a patient with prostate cancer. *Hum Gene Ther*. 1995;6:1417–1426.
8. Chung I, Peter ES, Crystal RG, et al. Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cell. *Cancer Gene Ther*. 1999;6:99–106.
9. Peng XY, Won JH, Rutherford T, et al. The use of the L-plastin promoter for adenoviral-mediated, tumor-specific gene expression in ovarian and bladder cancer cell lines. *Cancer Res*. 2001;61:4405–4413.
10. Zhang L, Akbulut H, Tang Y, et al. Adenoviral vectors with E1a regulated by tumor specific promoters are selectively cytolytic for breast cancer and melanoma. *Mol Ther*. 2002;6:386–393.
11. He T-C, Zhou S, DaCosta LT, et al. A simplified system for generating recombinant viruses. *Proc Natl Acad Sci USA*. 1998;95:2509–2514.
12. Becker TC, Noel RJ, Coats WS, et al. Use of recombinant adenovirus for metabolic engineering of mammalian cells. *Methods Cell Biol*. 1994;43Pt A:161–189.
13. Sloane DL, So OY, Leung R, et al. Cloning and functional expression of the cDNA encoding rat lanosterol 14-alpha demethylase. *Gene*. 1995;161:243–248.
14. Freshney RI. *Culture of Animal Cells: a Manual of Basic Technique*, 4th edn. New York, NY: Wiley-Liss; 2000:329–344.
15. Kievit E, Nyati MK, Ng E, et al. Yeast cytosine deaminase improves radiosensitization and bystander effect by 5-fluorocytosine of human colorectal cancer xenografts. *Cancer Res*. 2000;60:6649–6655.
16. Alemany R, Balague C, Curiel DT. Replicative adenoviruses for cancer therapy. *Nat Biotechnol*. 2000;18:723–727.

Vector Targeting of 5FU Chemotherapy for Colon Cancer

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ABSTRACT

In order to develop a less toxic and more effective treatment for colon cancer, we have replaced 5-fluorouracil (5FU) in the Saltz combination of CPT-11, folinic acid (FA) and 5FU chemotherapy by 5-fluorocytosine (5FC) and an adenoviral vector which carries the L-plastin (Lp) tumor specific promoter driven transcription unit encoding the cytosine deaminase (CD) gene linked to the E1A gene by an IRES element. We call this new form of treatment program "Genetic Saltz Therapy". The experimental results reported in this paper show that the Genetic Saltz Therapy has a statistically significant advantage in terms of increased response rate, response duration, survival and reduced toxicity when compared to animals treated with the conventional Saltz combination chemotherapy. Genetic Saltz Therapy is a concept that could be applied widely for many forms of cancer treatment.

INTRODUCTION

5FU is a component of most of the existing chemotherapy regimens for advanced carcinoma of the colon as well as the programs used for the adjuvant therapy for carcinoma of the colon and carcinoma of the breast (1). The use of the weekly schedule of CPT-11, folinic acid (FA) and 5-fluorouracil (5FU) combination chemotherapy (which is known as the Saltz regimen) is associated with a 15 month median survival in advanced colorectal cancer (1). One of the limiting factors of the Saltz regimen is its gastrointestinal toxicity. When 5FU is given at the maximal doses which are safe to administer systemically, it is usually considered to be toxic only for dividing cells through incorporation of the 5FU into DNA and binding of 5FU to thymidylate synthase (2-3). The failure of existing 5FU based chemotherapy in many advanced colon cancer patients may be due in part to the fact that less than 10% of colon cancer cells are proliferating at any given time and therefore most of the colon cancer cells escape control by 5FU. If it were possible to safely increase the levels of intravenously administered 5FU to those at which RNA is sufficiently substituted with 5FU to suppress protein synthesis (2), then one could kill non-dividing colon cancer cells as well as dividing cancer cells. Unfortunately, the dose increments of systemically administered 5FU that would be required to prevent protein synthesis and thereby kill non dividing colon cancer cells would generate unacceptable levels of toxicity to the normal cells of the bone marrow and gastrointestinal tract.

We have therefore proposed to use the L-plastin tumor specific transcriptional promoter to regulate the expression of the transcription units of an adenoviral vector which is selectively cytolytic to tumor cells, on the basis of the levels of the CD protein which it produces in vector infected tumor cells. The L-plastin promoter (4-5) has been shown to drive the expression of genes in tumor cells but not in normal cells (6-8). The Escherichia Coli or yeast cytosine deaminase (CD) gene (9-10) catalyzes the conversion of the relatively harmless drug, 5-Fluorocytosine (5FC), into the cytotoxic agent, 5FU. The levels of 5FU which are generated by the CD/5FC system (3) within tumor cells (>300 micromolar) are much higher than those possible when 5FU is systemically administered (5 micromolar). Adenoviral vectors carrying the CD gene driven by the L-plastin promoter have been shown in our laboratory to sensitize breast, ovarian and colon cancer cells to the effects of 5FC (6-7). We have also placed the gene for E1A, which is necessary for viral replication, downstream of the L-plastin promoter to create a vector which is selectively cytolytic to cancer cells (11). A vector that contains both the CD and E1A genes under control of the L-plastin promoter (AdLpCDIRESE1A) can be used to kill cancer cells through two mechanisms: vector replication within the tumor cells, and sensitization of the cancer cell infected by this vector to the effects of 5FC. This double gene vector has already been shown in our laboratory to have a tumor selective cytotoxic effect that is greater than vectors carrying either the CD or the E1A genes alone (12).

In this report, we have combined the AdLpCDIRESE1A vector with CPT-11, 5FC and FA chemotherapy to create a treatment called "Genetic Saltz Therapy", which was designed to make the conventional Saltz chemotherapy combination of CPT-11, 5FU and FA less toxic and more effective for the treatment of colon cancer (1). The results of the experiments which are summarized in this report show that "Genetic Saltz Therapy" is less toxic and much more effective in suppressing the growth of colon cancer and extending the survival of mice than is the conventional Saltz combination chemotherapy. On the basis of the results reported in this paper, it appears that vector targeting of chemotherapy represents a novel concept of chemotherapy that could reduce toxicity and improve the therapeutic outcome in advanced colon cancer as well as in the chemotherapy of many epithelial neoplasms

RESULTS

Properties of Cell Lines Which Relate to Sensitivity to Chemotherapy and Vector Infectability.

To evaluate the reasons for differences in response to the AdLpCDIRESE1A vector and chemotherapy, we studied the CE and TS activity of the human tumor cell lines. The CE activity of Ln-CaP (human prostate cancer), MCF-7 (human breast cancer) and Ovcar-5 (human ovarian cancer) cells were similar. However, the HTB-38 human colon carcinoma cells had 6 times more enzyme activity than the other cell lines (Table 1). The TS activity (15), which was expressed as the amount of ^3H_2O (fmol) formed in 1 minute per mg of protein, was lowest in the HTB-38 colon cancer cell line and highest in the Ovcar-5 ovarian cancer cell line (Table 1). To characterize the cell lines with respect to differences which might alter the infectibility of the target cell lines, we studied the expression of CAR, $alpha_v beta_3$ and $alpha_v beta_5$ integrin receptors on tumor cells (17). The percentage of $alpha_v beta_3$, $alpha_v beta_5$ integrin receptor as well as the CAR positive cells was measured by flow cytometry. The percentage of cells positive for CAR and $alpha_v beta_5$ integrin receptors varied among the tumor cell lines. More than half of the tumor cell lines have significant percentages of CAR, $alpha_v beta_3$ and $alpha_v beta_5$ integrin receptor positive cells (Table 3). According to these results, Ln-CaP cell appear to be the most sensitive cell line in terms of taking up the adenoviral vectors. The results suggest that the HTB-38 cell line should be infectable by the adenoviral vectors as well.

In Vitro Studies of Vector Infected Cell Lines.

Western blot analysis (see Fig. 1b) for E1A expression following vector infection and two days of incubation of all vector infected tumor cell lines showed bands specific for E1A polypeptides (35-46 kDa). No protein bands are visible from the control cells which were not exposed to E1A containing vectors. The steady state levels of 5FU generated in the medium from AdLpCD infected MCF-7 cells exposed to 5FC were greater than 340 micromolar. These levels are far in excess of the IC50 of the MCF-7 breast cancer cells (see Table I) and far above the 5 micromolar levels that are generated by systemic administration of 5FU. These levels are similar to those reported previously to result in the inhibition of protein synthesis (2) and thereby to the death of non-dividing cancer cells.

Specificity of expression of the L-plastin promoter driven vector transcription units.

In order to test if the L-plastin driven bicistronic CDIRESE1A transcription unit was expressed in a tumor specific manner in the AdLpCDIRESE1A vector infected cells (6-8), we injected either the AdLpCDIRESE1A or the AdCMVCDIRESE1A vectors into normal liver or subcutaneous tumor nodules and stained histological sections of the injected tissue for E1A expression The tumor nodules were positive for E1A whether injected by the AdLpCDIRESE1A or AdCMVCDIRESE1A vectors (Fig. 1c1 and Fig. 1c3) whereas the normal liver tissue was positive for E1A only following injection with the AdCMVCDIRESE1A (Fig. 1c2 and Fig. 1c4). These results show that the expression of the transgenes in the AdLpCDIRESE1A vector infected cells is tumor specific.

Effect of the AdLpCDIRESE1A vector on IC50 values of CPT-11 SN-38, and 5FU.

We tested if the IC50 of 5FU, CPT-11 and SN-35 was decreased by exposure of the test cells to the AdLpCDIRESE1A vector. When the AdLpCD vector plus 5FC and FA were added to the test cells at MOI 10, the IC50 value of CPT-11 decreased 65-2200 times (Table 2). When the AdLpCDIRESE1A replication competent vector plus 5FC and FA was added to the cells, the decrease in the CPT-11 IC50 was 5-20 times the decrease seen with the AdLpCD replication deficient vector. In contrast, when the AdLpCDIRESE1A vector plus FA without 5FC was combined with CPT-11, the decrease of the IC50 value of CPT-11 was between 7 and 200 fold (data not shown). These studies show that the in vitro activity of CPT-11 and SN-38 is potentiated by the addition of the AdLpCDIRESE1A vector. Moreover, this vector sensitization does not depend on the replication competency of the vector but on the presence of the CD protein and 5FC.

Replacement of 5FU in the conventional Saltz combination chemotherapy by intratumoral injection of the AdLpCDIRESE1A vector and in vivo 5FC ("Genetic Saltz" Therapy) increases the tumor response and decreases toxicity as compared to the conventional Saltz combination chemotherapy (Animal Model #1).

Response Studies. The growth of the HTB-38 colon cancer cell line in nude mice was suppressed more by intratumoral injection of the AdLpCDIRESE1A vector given in concert with ip 5FC, iv FA and iv CPT-11 chemotherapy (Group 1) than it was by conventional Saltz combination chemotherapy (Group 6) as shown in Fig.

2a. The duration of tumor response among the animals treated with the Genetic Saltz Therapy (AdLpCDIRESE1A/5FC/FA/CPT-11-Group 1) was statistically significantly longer than the duration of the response among animals treated with regimens not containing the AdLpCDIRESE1A/5FC combination (p<.0001).

<u>Survival Studies</u>. The mice treated with AdLpCDIRESE1A+5FC+FA+CPT-11 (the Genetic Saltz Therapy or Group 1) lived much longer than did the mice treated with conventional Saltz combination chemotherapy (Group 6) or the other control groups (Fig. 2b). We then tested for the effect of increasing the frequency of the AdLpCDIRESE1A vector injections from once a week to twice a week. A survival advantage was seen in this latter model but this advantage was lost by 12 weeks (data not shown). This suggested that additional cycles of therapy might be one way to increase the success of the outcome.

<u>Toxicity Studies</u>. Importantly, the mice given the conventional 5FU, FA and CPT-11 combination chemotherapy (Group 6) had statistically significantly more diarrhea and slightly more weight loss than that observed in mice treated with the Genetic Saltz Therapy, which involves the combination of AdLpIRESE1A/5FC/FA/CPT-11, as shown in Table 4.

<u>Response of the HTB-38 colon cancer cells to in vitro infection with the AdLpCDIRESE1A vector infection and in vivo 5FC/FA/CPT-11 chemotherapy (Animal Model #2)</u>.

To test if we could improve the outcome of the Genetic Saltz Therapy, we infected the HTB-38 cells with AdLpCDIRESE1A vector in vitro under conditions that would result in infection of 100% of the HTB-38 cancer cells prior to the injection of the tumor cells into the subcutaneous space of the test animals. Following subcutaneous injection of HTB-38 tumor cells which had been infected in vitro with either the AdLpCDIRESE1A vector, the AdLpCD vector, or the AdWT virus, we treated the mice with the programs outlined in Fig. 1c.

<u>Response Studies.</u> None of the mice treated with the Genetic Saltz Therapy (Group 1) exhibited regrowth of the tumor cells at the injected sites during the 5 months of follow-up whereas the other treatment groups showed regrowth of tumor after chemotherapy (Fig. 2c). In each of the groups in which the colon cancer cell line HTB-38 was infected in vitro with a replication competent vector (Groups 1 and 4) and in which in vivo 5FC (in the case of the AdLpCDIRESE1A) or 5FU (in the case of AdWT) was given, there was a statistically significant reduction in the tumor growth rates (Fig. 2c).

Survival Studies. There was a survival advantage of the Genetic Saltz Therapy (Group 1) as compared to the use of in vivo administration of the conventional Saltz combination chemotherapy (Group 5) as shown in Fig. 2d. When 5FC was deleted from the Genetic Saltz Therapy, the survival advantage of the Genetic Saltz Therapy over the conventional Saltz combination chemotherapy was lost (eg Group 3 in Fig.2d). These results indicated that the outcome of therapy depended on the conversion of 5FC to 5FU within the tumor cells.

DISCUSSION

We have tested if it is possible to increase the efficacy and decrease the toxicity of combination chemotherapy for advanced carcinoma of the colon by using a vector to target 5FU therapy to tumor cells and spare the normal cells of the body. The combination of the AdLpCDIRESE1A conditionally replication competent adenoviral vector with 5FC, CPT-11 and FA chemotherapy, which is called the "Genetic Saltz Therapy", is statistically significantly superior to the conventional Saltz combination chemotherapy (5FU, FA and CPT-11) with respect to tumor response and survival. These effects may be due to the high levels of 5FU generated by the AdLpCDIRESE1A vector/5FC treatment within tumor cells that are not possible to achieve using systemic administration of 5FU chemotherapy. In addition, we show that the toxicity of the Genetic Saltz Therapy is statistically significantly less than that seen with the conventional Saltz combination chemotherapy.

Since its synthesis in 1957, 5-FU has been the primary chemotherapeutic agent in advanced colorectal carcinoma and in the adjuvant therapy of carcinomas of the colon and breast. However, the median survival of the patients with advanced colon cancer produced by the best 5-FU regimens has been between 12-15 months (18). Recently, Douillard JY et al reported increased response rates with use of a weekly and biweekly schedule of CPT-11 combined with two different 5-FU regimens including high dose FA (19). Saltz et al reported increased response rates and prolongation of survival with the combination of a weekly schedule of CPT-11 and 5-FU plus low dose FA when compared to 5-FU, FA regimens, which are similar to those used in the CPT-11 arms (20-21).

Mice which were given the Genetic Saltz Therapy had a tumor volume which was almost ¼ of that seen in the mice treated with the conventional Saltz combination chemotherapy ($p=.0001$) at the end of the first month. The mice given the Genetic Saltz Therapy also had statistically significantly prolonged survival compared to the mice given the conventional Saltz combination chemotherapy ($p=.0004$). The addition of FA to the AdLpCDIRESE1A vector and 5-FC (Genetic Saltz Therapy without CPT-11) also enhanced tumor control and survival more than the conventional Saltz combination chemotherapy.

Animal model #2 was designed to test the efficacy of the vector under conditions which permitted infection of 100% of the tumor cells. This was accomplished by infecting the tumor cells with either CD carrying vectors or wild type adenovirus before subcutaneous inoculation of the tumor cells into the test mice. All of the mice given the Genetic Saltz Therapy were free of tumor nodules, whereas 6/7 of the mice given AdLpCDIRESE1A+CPT-11(iv)+FA(iv), which is the Genetic Saltz Therapy without 5FC, developed tumor nodules.

One of the major limiting factors of the conventional Saltz combination chemotherapy is its gastrointestinal toxicity. Importantly, there was a statistically significantly decreased incidence of diarrhea in the animals treated with the Genetic Saltz Therapy as compared with conventional Saltz combination chemotherapy. This reduction in toxicity and increase in efficacy of the regimen was generated by using conditionally replication competent adenoviral vectors which are tumor specific in the delivery of 5FU to cancer cells, thus sparing the normal tissues of the body from the toxicity of the regimen.

The results of these studies suggest that the combination of AdLpCDIRESE1A and 5-FC system with CPT-11 and FA is more effective and less toxic than the traditional combination of CPT-11, 5-FU and FA (conventional Saltz combination chemotherapy). Because of the limited number of tumor cells infected when this vector is injected intratumorally, the goal of clinical translation of the Genetic Saltz Therapy will be feasible when the vector has been engineered so that it only infects tumor cells and tumor vascular endothelial cells. In that case, the vector will be suitable for administration in the bloodstream and under these conditions, it is possible that a far greater number of tumor cells can be accessed by the AdLpCDIRESE1A vector. Work towards this goal is ongoing presently in our laboratory.

METHODS

Cells and reagents.

All cell lines were purchased from the American Type Culture Collection (ATCC) except for the human epithelial ovarian cancer cell line (Ovcar-5) which was obtained from Dr. Thomas C. Hamilton of Fox Chase Cancer Center, Philadelphia, PA. All drugs and chemicals were purchased from Sigma (St Louis, Missouri). 2'-Deoxyuridine 5'-monophosphate and diammonium salt, [5-^{3}H] (15.0 Ci/mmol) were purchased from Moravek (Brea, California); IgG-FITC was purchased from eBioScience (San Diego, California). Alpha$_v$beta$_5$ and alpha$_v$beta$_3$ monoclonal antibodies were purchased from Covance (Richmond, California). Mouse anti-CAR antibody was kindly provided by Dr. Robert W. Finberg of University of Massachusetts Medical School. Wild type Adenovirus type 5 (Ad5WT) was purchased from the ATCC. The construction of the following vectors: AdLpE1A, AdCMVE1A, AdLpCD, AdLpCDIRESE1A, and AdCMVCDIRESE1A, has been described in previous publications from our laboratory (6-8, 12).

Carboxylesterase and Thymidylate synthase (TS) activities of tumor cells.

Carboxylesterase (CE) activity of all cell lines was assessed by measuring the hydrolytic conversion of paranitrophenolic acid (pNPA) to para-nitrophenol (pNP) which is catalyzed by CE (14). TS activity of tumor cells was measured according a tritium-release assay as described previously (15). The incubations were performed in quadruplicate.

Immunofluorescent Analysis.

The level of expression of coxackie-adenoviral receptor (CAR), alpha$_v$beta$_3$ and alpha$_v$beta$_5$ integrin receptors on tumor cells was measured as described in previous publications (7, 8, and 12) using an anti-mouse IgG-FITC antibody. Normal hepatic tissue and subcutaneous tumor nodules caused by inoculating HTB-38 cells infected with AdLpCDIRESE1A or AdCMVCDIRESE1A vectors were studied for the expression of E1A protein by immunofluorescent staining (Vector M.O.M. Immunodetection Kit, Burlingame, California) using Adenovirus type 5E1A antibody from NeoMarkers (Fremont, California).

Effect of vectors on the IC50 of 5 Fluorouracil, CPT-11, SN-38.

$1\text{-}5 \times 10^4$ cells/well were plated in 96-well plates in 100ul of culture medium. Following an overnight incubation, fresh medium supplemented with varying amounts of the test drug were added: for 5-FU, from 0.05uM to 410 uM-14 different concentrations; for CPT-11, from 5 nanomolar to 1.3 mM-10 different concentrations; and for SN-38, from 10 picomolar to 10 micromolar-7 different concentrations. After a 72-hour incubation period with drug, the medium was exchanged for fresh medium without drug. The next day, 10 ul of MTT reagent as provided in the commercial kit (ATCC, Manassas, VA, USA) were added to each well containing cells. Following an incubation period of 24 hours, which was continued until a purple precipitate was visible at 37°C, the plates were then incubated further overnight at room temperature. The results were expressed as the average percentage (in quadruplicate) of the population of cancer cells which were present before treatment which were left surviving at any time point.

Animal model #1.

3×10^6 HTB-38 colon cancer cells were injected subcutaneously into female nude/nude mice (4-6 wks of age). 1×10^8 pfu of the AdLpCDIRESE1A vector or the AdLpCD vector were injected intratumorally into subcutaneous nodules (50 mm^3) of the HTB-38 colon cancer cells which developed from the subcutaneous injection of tumor cells in female nude/nude mice (Fig. 1a). The vector or phosphate buffered saline control injections into the tumor nodules were repeated on days 1, 8, 15 and 22. All the drugs were given to test mice at doses which were equivalent to human doses (5FC 500 mg/kg/day i.p. for 10 days; 5FU 150 mg/kg, i.v. days 1, 8, 15, and 22; FA 6 mg/kg/day, i.v. daily starting on the day of intratumoral vector injection for the vector groups and on days 1, 8, 15, and 22 for the CPT-11+5-FU+FA group; CPT-11 40 mg/kg, i.v. days 1, 8, 15, and 22 (16). A total of two complete cycles of therapy (each six weeks long) were given to all animals. Tumor volumes were measured every 2 days (7-8). Data are represented as the mean change in tumor size relative to the tumor size at the beginning of treatment of each animal. Animals were evaluated for toxicities of the treatment regimens every day. The following parameters for toxicity were measured daily: activity, skin color, hunching, fur status, and the cage bedding was inspected for residues of diarrhea daily. The weight of mice was measured three times a week. A separate set of experiments was carried out in which the vector was injected twice a week (instead of the once a week described above), and the tumor dose injected was 2×10^5 cells instead of 3×10^6 cells.

Animal model #2.

In order to test the efficacy of the vector in a way which was not limited by diffusion and spread of the vector from the intratumoral injection sites of the vector particles to each of the tumor cells in the tumor nodule, we first incubated the HTB-38 cells in vitro at 30 MOI (100% of the HTB-38 cells were previously shown to be infected at MOI 30) with either the AdLpCDIRESE1A vector or the wild type adenovirus (AdWT) for 60 minutes. Following subcutaneous injection of the vector infected tumor cells ($5x10^5$ cells/mouse), we administered the drugs at the same doses used in animal experiment #1 to the assigned groups (Fig 2c). Two cycles of therapy were given in all groups except group 1. In group 1, ip 5-FC injections were given only in the first and second week because no tumor appeared.

Statistical analysis.

IC50 values were calculated according to the median effect principle. The differences among the results of the various groups were compared by the Student's t test. One-way ANOVA (with LSD post-hoc comparisons) and Mann-Whitney tests were used for the comparison of tumor volumes. Tumor growth rates were evaluated by regression analysis. Survival analyses were performed according to the Kaplan-Meier method and the log-rank test was used for survival comparisons.

ACKNOWLEDGEMENTS

The authors would like to acknowledge support to the SKCC by Sidney Kimmel and the following grants to Albert Deisseroth: the Breast Cancer Research Foundation, The US Army Breast Cancer Research Program (Grant # DAMD 17999457), The Bush Leukemia Research Foundation, The Anthony Dewitt Frost Melanoma Research Fund, The Audrey Demas Melanoma Research Fund, and support to our cancer center from Sidney Kimmel. The authors would like to acknowledge Dr. Fikri Icli of Ankara University for his contributions to the discussions that let to the idea of vector targeting chemotherapy. The authors would also like to acknowledge support to Hakan Akbulut from the Turkish Academy of Sciences in the framework of the Young Scientist Award Program (EA- TUBA-GEBIP/2001-1-1) and the National Cancer Institute of United States of America.

REFERENCES

1. Saltz, L.B., *et al.* Irinotecan plus fluorouracil/leucovorin for metastatic colorectal cancer: a new survival standard. *Oncologist* **6**, 81-91 (2001).
2. Armstrong, R.D., Lewis, M., Stern, S.G., & Cadman, E.C. Acute effect of 5FU on cytoplasmic and nuclear dihydrofolate reductase mRNA metabolism. *J. Biol. Chem.* **261**, 7366-7371 (1986).
3. Garcia-Sanchez, F., Pizzorno, G., Krause, D.S., Liang, J., Adams, E., Leffert, J.J., Fu, S.Q., Hanania, E., Giles, R., Crystal, R.G. & Deisseroth, A.B. Cytosine deaminase adenoviral vecgtor and 5-fluorocytosine selectivity reduce breast cancer cells 1 million-fold when they contaminate hematopoietic cells: a potential purging method for autologous transplantation. *Blood* **92**, 672-682 (1998).
4. Lin, C.S., Chen, Z.P., Park, T., Gosh, K., & Leavitt, J. Characterization of the human L-plastin gene promoter in normal and neoplastic cells. *J. Biol. Chem. 268*, 2793-2801 (1993).
5. Lin, C.S., Park, T., Chen, Z.P., & Leavitt, J. Human plastin genes. *J. Biol. Chem.* **268**, 2781-2792 (1993).
6. Chung, I., Peter, E.S., Crystal, R.G., Pizzorno, G., & Deisseroth, A. Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not in normal mesothelial cell. *Cancer Gene Therapy* **6**, 99-106 (1999).
7. Peng, X.Y., Rutherford, T., Won, J.H., Pizzorno, G., Zelterman, D., Sapi, E., Kaczinski, B., Leavitt, J., Crystal, R., and Deisseroth, A. The use of the L-plastin promoter for adenoviral-mediated, tumor-specific gene expression in ovarian and bladder cancer cell lines. *Cancer Res.* **61**, 4405-4413 (2001).
8. Zhang, L., Akbulut, H., Tang., Y.C., Peng, X.Y., Pizzorno, G., Sapi, E., Manegold, S., and Deisseroth, A., et al. Adenoviral vectors with E1a regulated by tumor specific promoters are selectively cytolytic for breast cancer and melanoma. *Molecular Therapy* **6**, 386-393 (2002).
9. Nettelbeck, D.M., Jerome, V., & Muller, R. Gene therapy: designer promoters for tumor targeting. Trends in Genetics 16, 174-181 (2000).
10. Hirschowitz, E.A., *et al.* In vivo adenovirus-mediated gene transfer of the Escherichia coli cytosine deaminase to human colon carcinoma-derived tumor induces chemosensitivity to 5-fluorocytosine. *Human Gene Therapy* **6**, 1055-1063 (1995).

11.Graham, F.L., & Prevec, L. Manipulation of adenovirus vectors, in Methods in Molecular Biology, Gene Transfer and Expression Protocols, Vol 7 (ed. Murray EJ) 109 (Humana, Clifton, MJ, 1991)

12.Akbulut, H., Zhang, L., Tang, Y., & Deisseroth, A. The cytotoxic effect of replication competent adenoviral vectors carrying L-plastin promoter regulated E1A and cytosine deaminase genes in cancers of the breast, ovary and colon. *Cancer Gene Therapy* **10**, 388-395 (2003).

13.He, T.-C., *et al.* A simplified system for generating recombinant viruses. *Proc. Natl. Acad. Sci. USA* **95**, 2509-2514 (1998).

14.van Ark-Otte, J., *et al.* Determinants of CPT-11 and SN-38 activities in human lung cancer cells. *Br. J. Cancer* **77**, 2171-2176 (1998).

15.Beck, A., *et al.* Role for dihydropyrimidine dehydrogenase and thymidylate synthase in tumour sensitivity to fluorouracil. *Eur. J. Cancer* **30**, 1517-1522 (1994).

16.Freireich, E.J., Gehan, E.A., Rall, D.P., Schmidt, L.H., & Skipper, H.E. Quantitative Comparison of Toxicity of Anticancer Agents in Mouse, Rat, Hamster, Dog, Monkey, and Man. Cancer *Chemotherapy Reports* **50**, 219-244 (1966).

17.Wickham, T.J., Mathias, P., Cheresh, D.A., & Nemerow, G.R. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell* **73**, 309-319 (1993).

18.The Advanced Colorectal Cancer Meta-Analysis Project: Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: evidence in terms of response rate. *J. Clin. Oncol.* **10**, 896-903 (1992).

19.Douillard, J.Y., *et al.* Irinotecan combined with fluorouracil compared with fluorouracil alone as first line treatment for metastatic colorectal cancer: a multicenter randomized trial. *Lancet* **355**, 1041-7 (2000).

20.Saltz, L.B., *et al.* Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer: Irinotecan Study Group. *N. Engl. J. Med.* **343**, 905-4 (2000).

21.Saltz, L., *et al.* Cetuximab (IMC-C225) plus irinotecan (CPT-11) is active in CPT-11refractory colorectal cancer (CRC) that expresses epidermal growth factor receptor (EGFR). *Proceedings of ASCO* **20**:3a(Abstract No:7, (2001).

FIGURE LEGENDS

Figure 1. Tumor specific adenoviral vectors.

Figure 1a. The maps of the therapeutic transcription units in adenoviral vectors. CD=cytosine deaminase gene; IRES=intraribosomal entry site; E1A= adenoviral replication gene; CMV=cytomegalovirus; Lp=L-plastin.

Figure 1b. Western blotting of E1A polypeptides produced in vector infected HTB-38 cells. The E1A region encodes a series of related proteins (35–46kDa).

Figure 1c. Tumor specific expression of the E1A gene following injection with vectors carrying the CMV or L-plastin promoters. In this study, green color indicates expression of E1A peptides.

Panel 1c1. In the normal liver injected with the AdLpCDIRESE1A vector, there is no green fluorescence of E1A polypeptides.

Panel 1c2. In the tumor nodule injected with the AdLpCDIRESE1A vector, the green fluorescence is detectable showing that the E1A polypeptides (which are stained green) are produced by the L-plastin driven E1A gene and are present with the nuclei of the tumor cells which are stained red.

Panel 1c3. The liver was injected with the AdCMVCDIRESE1A vector. The nuclei of the liver cells are stained blue while the E1A polypeptides are stained green in the cells injected with the vector carrying the tumor non-selective CMV promoter. These data show that the normal cells can be infected by the vector and can express E1A peptides in the presence of the CMV promoter.

Panel 1c4. The tumor nodule which was injected with the AdLpCDIRESE1A vector also showed expression of the E1A polypeptides.

Figure 2. Tumor Volumes and Survival Curves of the Assigned Treatment Groups in Animal Models #1 and #2.

Panel A of Figure 2. Tumor Response of Colon Cancer in Mice Following Treatment in Animal Model #1. The effect of the intratumoral injection of the AdLpCDIRESE1A vector along with intraperitoneal 5-FC daily for 10 days and one day of intravenous FA and CPT-11 ("Genetic Saltz" Therapy-Group 1) on the growth of HTB-38 cells is greater than that of the conventional Saltz combination chemotherapy (Group 6) and of other treatment groups ($p<.05$).

Panel B of Figure 2. Survival of Mice Following Treatment in Animal Model #1. The effect of the intratumoral injection of the AdLpCDIRESE1A vector along with 10 days of intraperitoneal 5-FC and one day of intravenous FA and CPT-11 ("Genetic Saltz" Therapy-Group 1) on prolongation of survival of mice carrying subcutaneous nodules of HTB-38 cells is greater than that of the conventional Saltz combination chemotherapy-Group 6 ($p<.01$).

Panel C of Figure 2. Tumor Response of Colon Cancer in Mice Following Treatment in Animal Model #2. The effect of in vitro infection of HTB-38 cells with the AdLpCDIRESE1A vector along with 10 days of intraperitoneal 5FC and one day of intravenous FA and CPT-11 ("Genetic Saltz" Therapy-Group 1) on tumor growth is greater than that of the conventional Saltz combination chemotherapy-Group 5 ($p<.05$).

Panel D of Figure 2. Survival of Mice Following Treatment in Animal Model #2. The effect of in vitro infection of HTB-38 cells with the AdLpCDIRESE1A vector along with 10 days of intraperitoneal 5-FC and one day of intravenous FA and CPT-11 ("Genetic Saltz" Therapy-Group 1) on prolongation of survival is greater than that of the conventional Saltz combination chemotherapy-Group 5 ($p=.0001$).

Table 1. CE (Mean ± standard error) and TS activity (Mean ± standard error) of the tumor cell lines and the IC50 values of the 5-FU, CPT-11 and SN-38 in these cell lines

Tumor cells	TS activity*	5-FU_{IC50} (uM)	CE activity**	$CPT11_{IC50}$ (uM)	$SN\text{-}38_{IC50}$ (uM)	IC50 CPT11/SN-3
Ln-CaP	6813±1914	0.1±0.01	0.3±0.02	0.9±0.3	$4.2 \pm 0.1 \times 10^{-5}$	2.1×10^{4}
MCF-7	5441±411	0.7±0.1	0.3±0.01	14.2±3.	$5 \pm 1.0 \times 10^{-4}$	2.8×10^{4}
Ovcar-5	7954±1247	2.8±0.7	0.3±0.02	21.4±4.9	$4.6 \pm 2.2 \times 10^{-1}$	4.7×10^{1}
HTB-38	4342±469	1.6±0.3	1.99±0.11	5.8±1.4	$5.1 \pm 0.5 \times 10^{-5}$	1.1×10^{5}

*fmol/min/mg.protein;

**mU/mg protein

Table 2. The IC50 values of CPT-11 and SN-38 in tumor cells following exposure to the AdLpCD or AdLpCDIRESE1A vectors at 10 MOI.

Tumor cells	$CPT\text{-}11_{IC50}$ (uM)		$SN\text{-}38_{IC50}$ (uM)	
	AdLpCD	AdLpCD-IRESE1A	AdLpCD	AdLpCD-IRESE1A
Ln-CaP	$3.5 \pm 1.4 \times 10^{-3}$	$2.5 \pm 0.9 \times 10^{-4}$	$6.8 \pm 5.14 \times 10^{-12}$	$3.5 \pm 1.2 \times 10^{-12}$
MCF-7	$6.2 \pm 2.3 \times 10^{-3}$	$1.4 \pm 1.5 \times 10^{-3}$	$2.8 \pm 1.8 \times 10^{-8}$	$3.9 \pm 1.6 \times 10^{-10}$
Ovcar-5	$2.8 \pm 1.1 \times 10^{-2}$	$8.2 \pm 4.4 \times 10^{-3}$	$6.1 \pm 3.5 \times 10^{-8}$	$5.1 \pm 1.8 \times 10^{-8}$
HTB-38	$8.8 \pm 1.7 \times 10^{-3}$	$4.3 \pm 2.3 \times 10^{-3}$	$7.8 \pm 4.2 \times 10^{-12}$	$8.2 \pm 1.3 \times 10^{-12}$

Table 3. The percentage of tumor cells positive for the CAR, Alpha$_v$Beta$_3$, and Alpha$_v$Beta$_5$ receptors as measured by FACS analysis.

Cell lines	CAR (%)	AvB5* (%)	AvB3** (%)
Ln-CaP	82.0±13.8	64.1±0.8	39.3±1.6
MCF-7	37.5±7.5	94.7±3.8	19.1±0.8
Ovcar-5	63.6±19.6	88.2±4.6	46.8±1.4
HTB-38	77.9±17.6	65.6±5.8	29.5±2.2

*AvB5=Alpha$_v$Beta$_5$

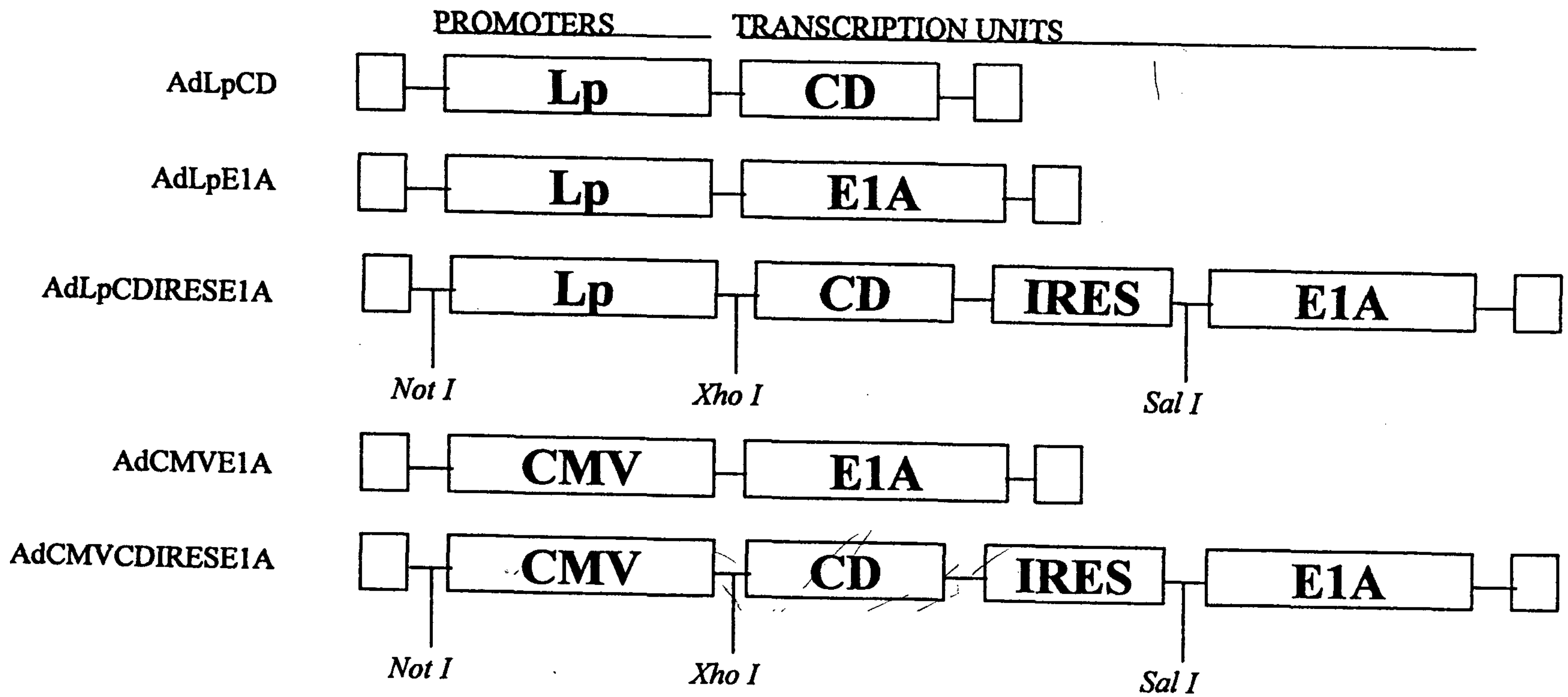
**AvB3=Alpha$_v$Beta$_3$

Table 4. Common toxicities seen in the treatment groups of Animal Model #1 (Percent of the total cycles in the first four weeks of treatment).

Groups	Treatment	Diarrhea	Weight**
Group #1	AdLpCDIRESE1A+5FC+FA+CPT-11	2.5	10.4±1.0
Group #2	AdLpCD+5FC+FA+CPT-11	0	3.0±0.6
Group #3	AdLpCDIRESE1A+5FC	0	8.5±0.6
Group #4	AdLpCDIRESE1A+5FC+FA	0	4.1±0.5
Group #5	AdLpCD+5FC+FA	0	6.7±0.6
Group #6	CPT-11+5FU+FA	32.5*	3.0±0.6
Group #7	AdLpCDIRESE1A+FA+CPT-11	2.5	4.8±0.7
Group #8	Control	0	8.6±0.6

- *The mice in the Saltz regimen (CPT-11+5-FU+FA) which is Group 6 had significantly more episodes of diarrhea (grade 1 or more) than the other groups (p=.001)
-
- **Denotes the percent change of weight during the first cycle of the treatment.

Fig. 1a



PROMOTERS
TRANSCRIPTION UNITS
AdLpCD
Lp
CD
AdLpE1A
Lp
E1A
AdLpCDIRESE1A
Lp
CD
IRES
E1A
Not I
Xho I
Sal I
AdCMVE1A
CMV
E1A
AdCMVCDIRESE1A
CMV
CD
IRES
E1A
Not I
Xho I
Sal I

Fig. 1b

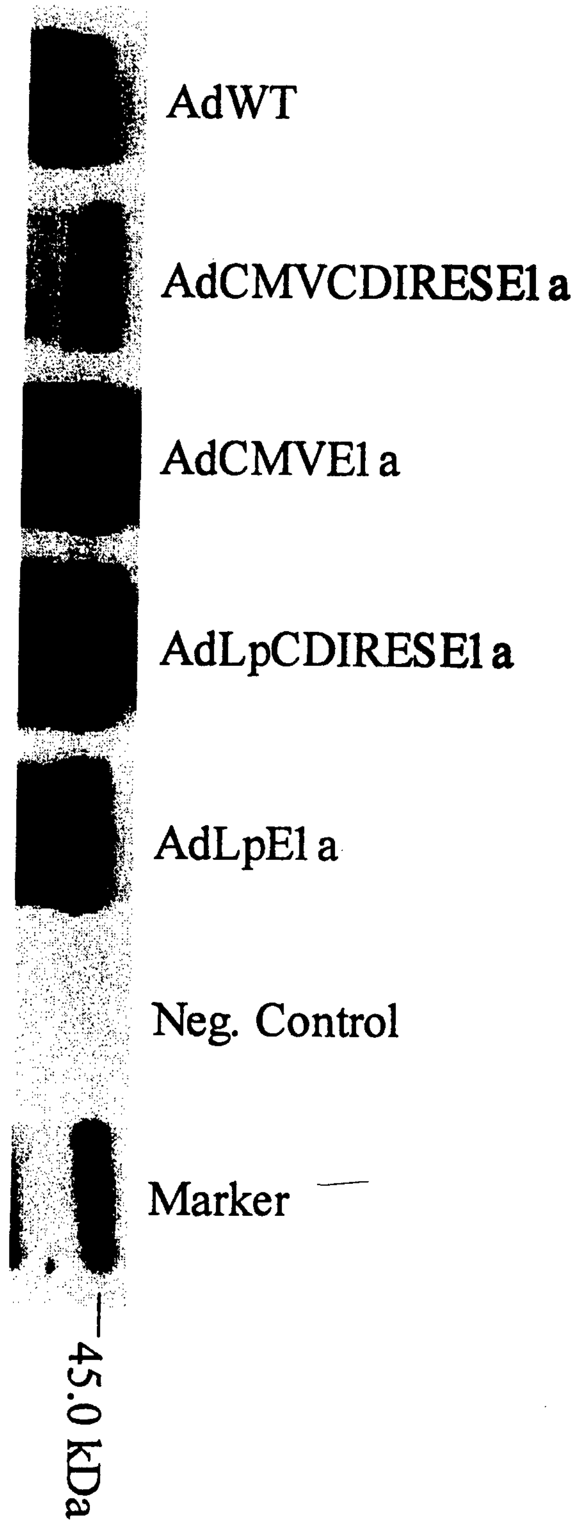

AdWT
AdCMVCDIRESE1 a
AdCMVE1 a
AdLpCDIRESE1 a
AdLpE1 a
Neg. Control
Marker
45.0 kDa

Fig. 1c1

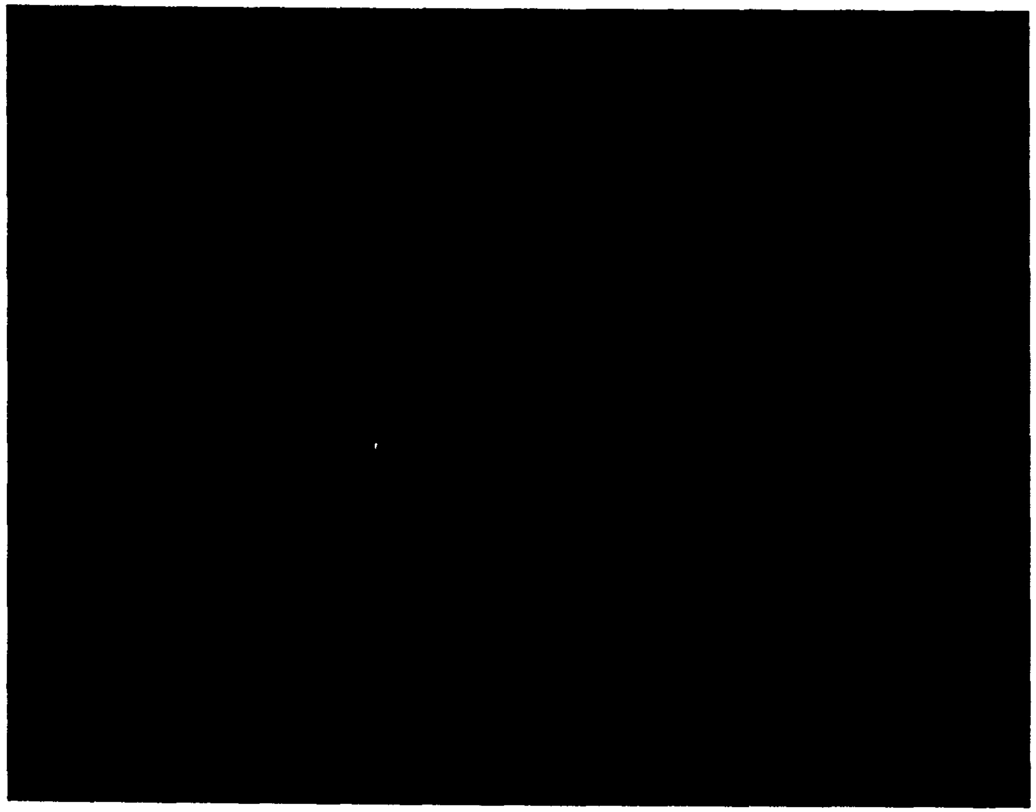


Fig. 1c2

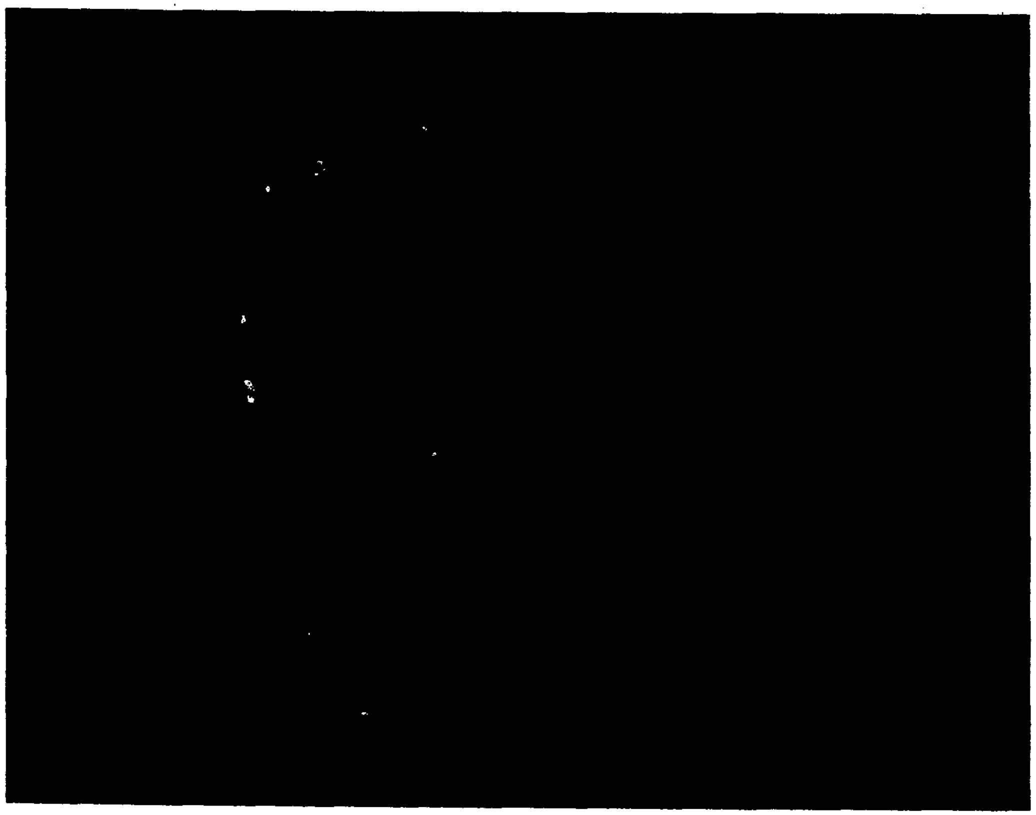
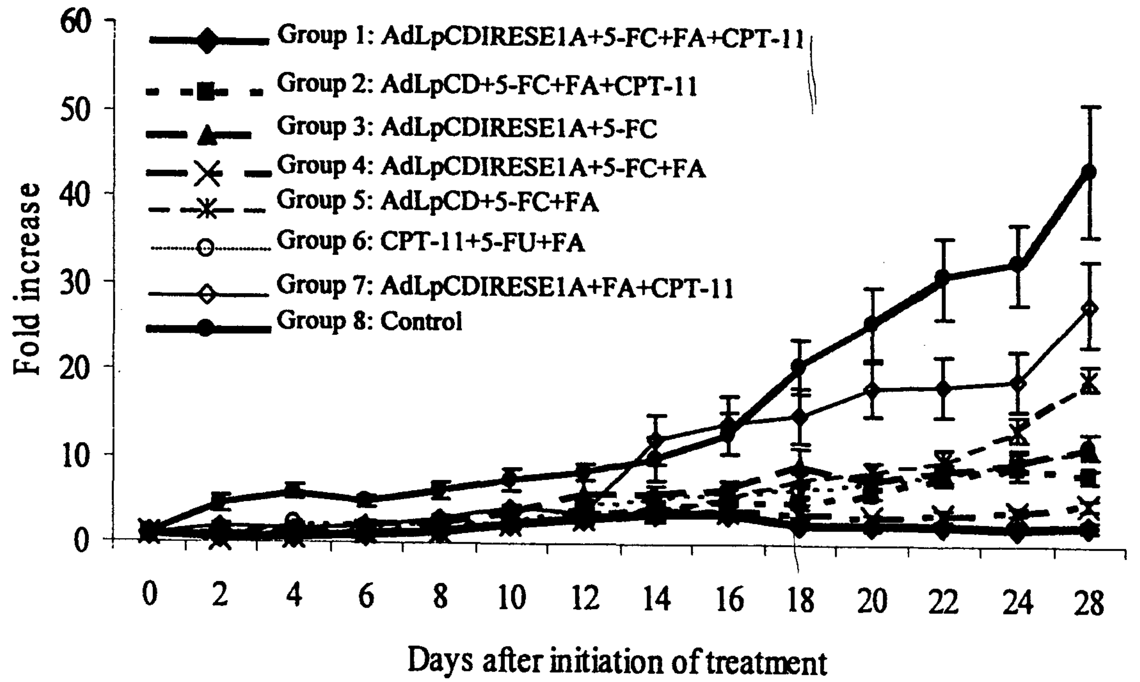


Fig. 1c3

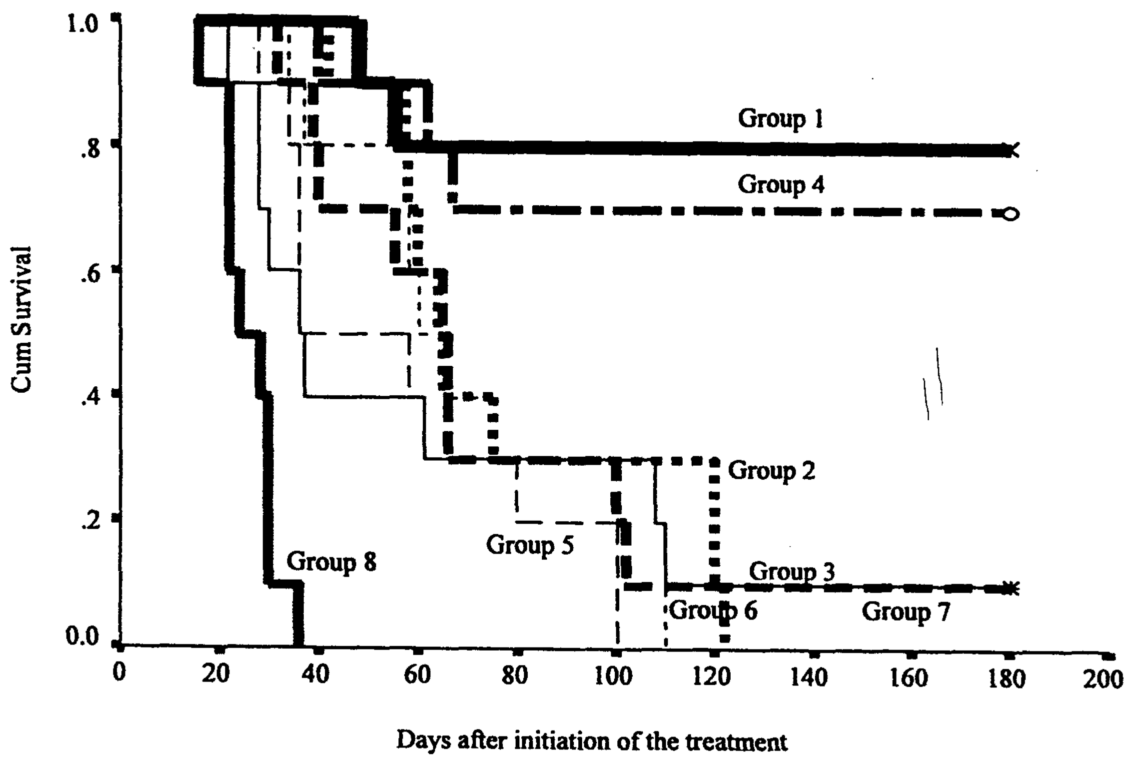
Fig. 1c4

Fig 2a



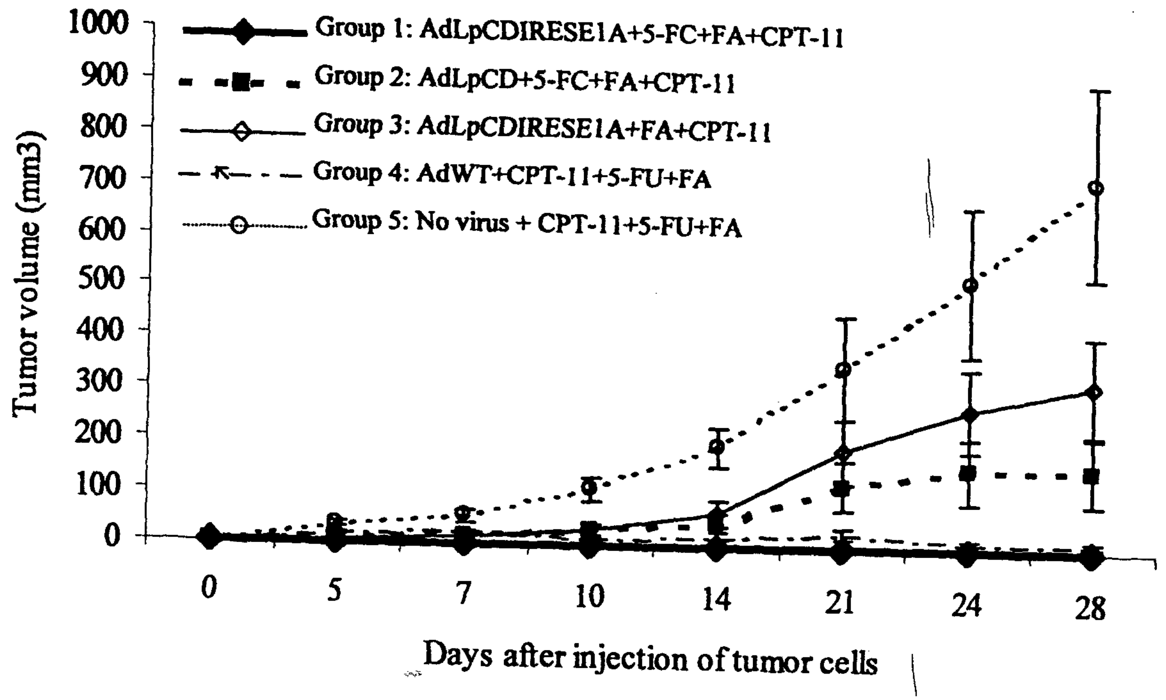
Group 1: AdLpCDIRESE1A+5-FC+FA+CPT-11
Group 2: AdLpCD+5-FC+FA+CPT-11
Group 3: AdLpCDIRESE1A+5-FC
Group 4: AdLpCDIRESE1A+5-FC+FA
Group 5: AdLpCD+5-FC+FA
Group 6: CPT-11+5-FU+FA
Group 7: AdLpCDIRESE1A+FA+CPT-11
Group 8: Control
Fold increase
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
16
18
20
22
24
28
Days after initiation of treatment

Fig. 2b



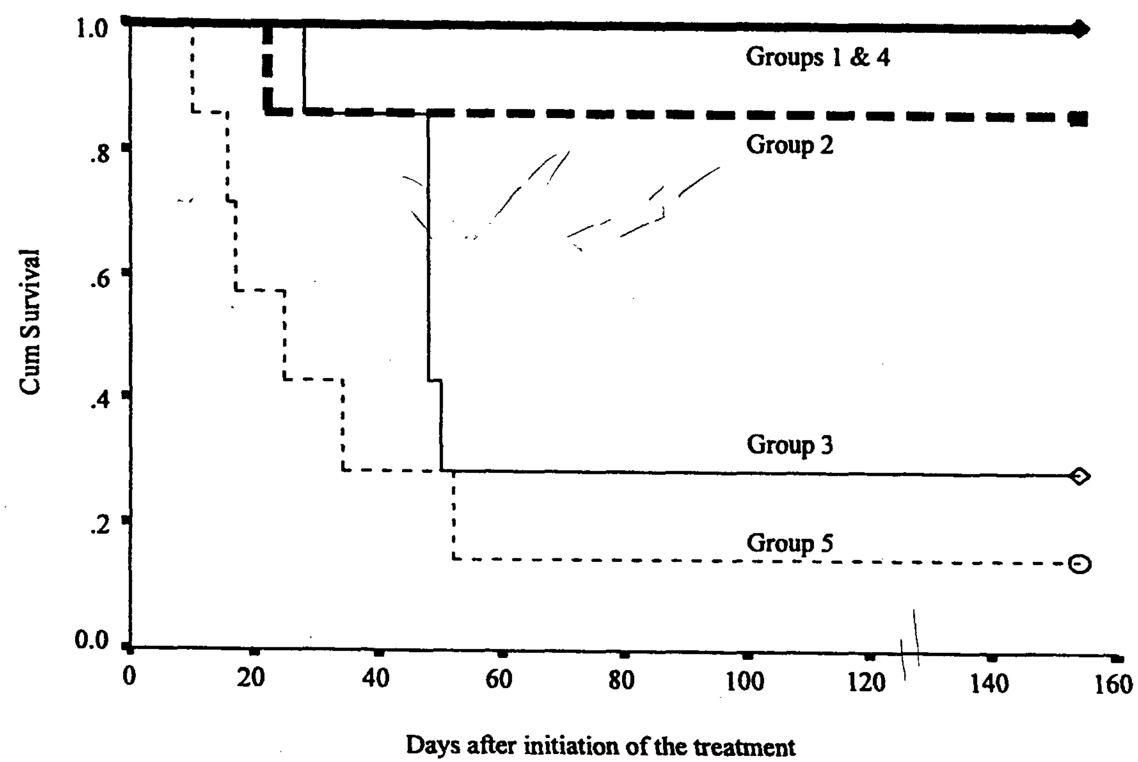
Cum Survival
1.0
.8
.6
.4
.2
0.0
0
20
40
60
80
100
120
140
160
180
200
Group 1
Group 4
Group 2
Group 5
Group 8
Group 3
Group 6
Group 7
Days after initiation of the treatment

Fig. 2c



Tumor volume (mm3)
1000
900
800
700
600
500
400
300
200
100
0
Group 1: AdLpCDIRESE1A+5-FC+FA+CPT-11
Group 2: AdLpCD+5-FC+FA+CPT-11
Group 3: AdLpCDIRESE1A+FA+CPT-11
Group 4: AdWT+CPT-11+5-FU+FA
Group 5: No virus + CPT-11+5-FU+FA
0
5
7
10
14
21
24
28
Days after injection of tumor cells

Fig. 2d



Groups 1 & 4
Group 2
Group 3
Group 5
Cum Survival
1.0
.8
.6
.4
.2
0.0
0
20
40
60
80
100
120
140
160
Days after initiation of the treatment