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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.

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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

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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 $\mu$g/ml), insulin (5 $\mu$g/ml), epidermal growth factor (10 ng/ml) and bovine pituitary extract (26 $\mu$g/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]

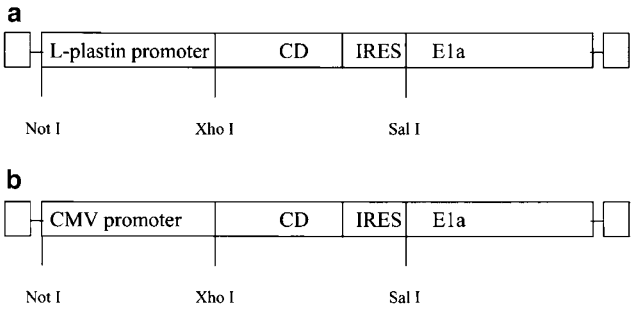



**Figure 1** The map of newly constructed conditionally replication-competent adenoviral vectors. E1a is transcriptionally coupled to the cytosine deaminase (CD) gene by an IRES in both vectors. In the AdLpCDIRESE1a vector (**a**), the bicistronic transcription unit is driven by the L-plastin promoter. 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 $\mu$M 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 $2 \times 10^5$ cells/well into six-well plates. After 24 hours, sufficient 5-FC was added to the cultures to generate a 5FC concentration of 500 $\mu$M. 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 ($3 \times 10^6$) 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 $\times$ (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

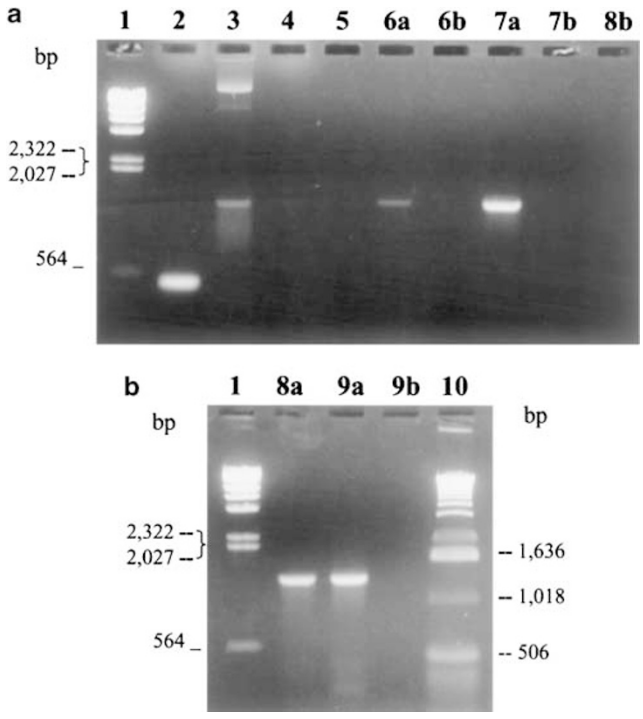



**Figure 2** Expression of CD gene in MCF-7 cells. Following a period of incubation of between 12 and 16 hours, the cells infected with AdLpCD, AdLpCDIRESE1a and AdCMVCDIRESE1a were trypsinized and washed with PBS. cDNA was then generated using the primers specific for the CD coding transcripts from 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 *Hind*III 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 $\beta$-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 Ad-LpCDIRESE1a 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

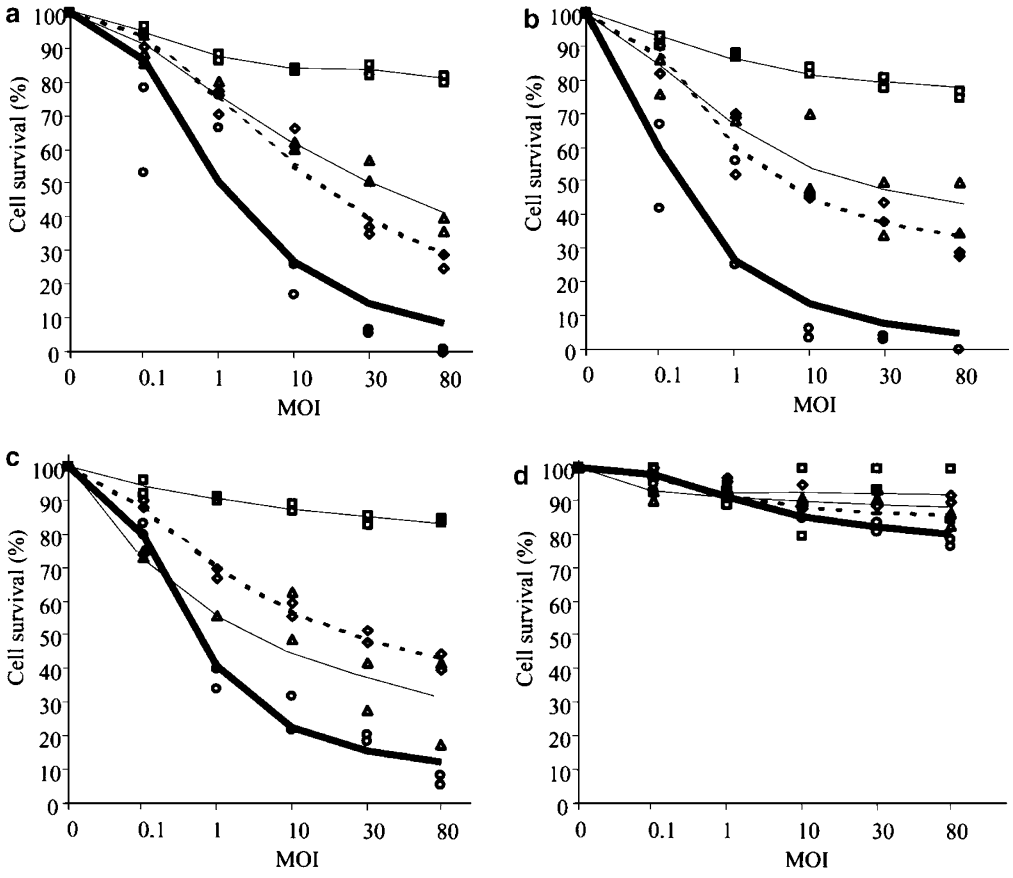



**Figure 3** Results of an *in vitro* cytotoxicity test. The tumor cell lines (**a**: MCF-7 cells; **b**: HTB-38 cells; **c**: Ovcar-5 cells), and explants of normal human mammary epithelial cells (**d**) were seeded in duplicate at a density of 200,000 cells/well in a six-well plate. They were then 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 $\mu$M 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 $\mu$M. 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

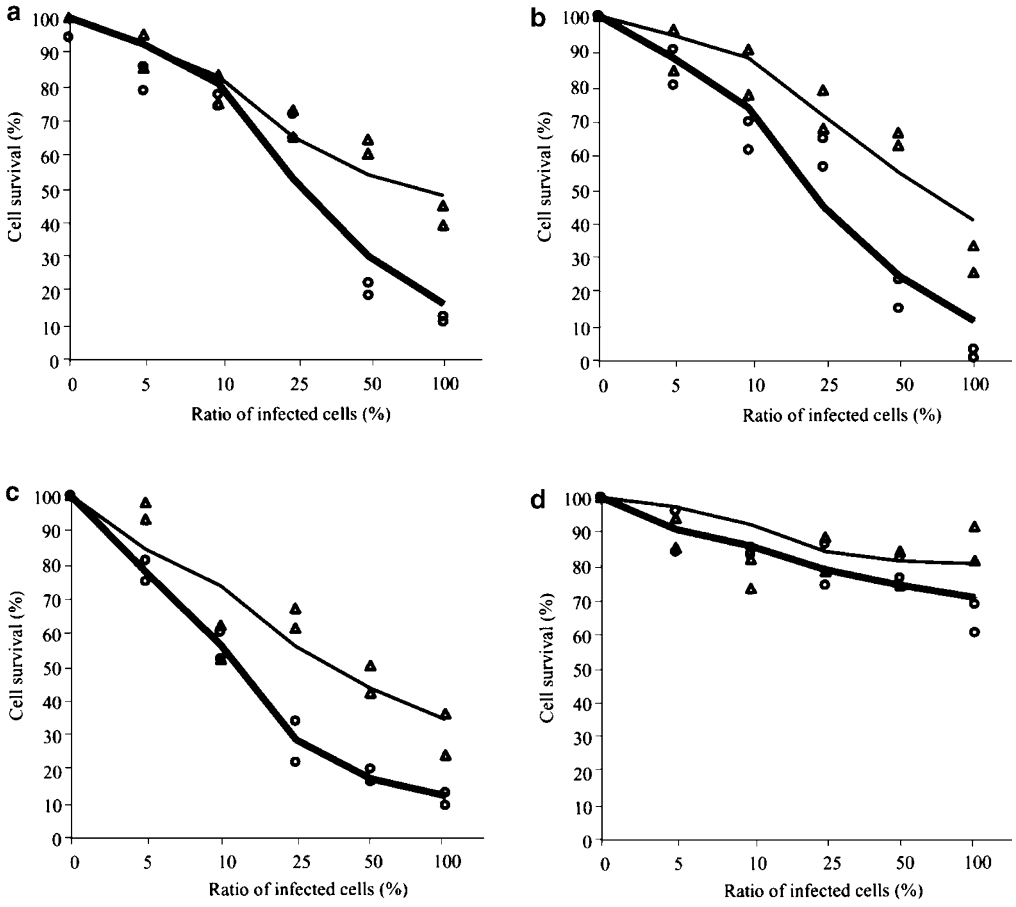



**Figure 4** Results of by-stander effect assay. The MCF-7 (**a**), the HTB-38 (**b**), the Ovcar-5 (**c**) and the HMEC (**d**) cell lines were infected at an MOI of 80 with the AdLpCDRESE1a and AdLpCD vectors. The infected cells were mixed with uninfected ones in varying ratios of 0, 5, 10, 25, 50 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 $\mu$M 5FC for 4 days. The percentage of surviving cells with AdLpCDIRESE1a+5FC (–△–); AdLpCD+5FC (–○–) 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

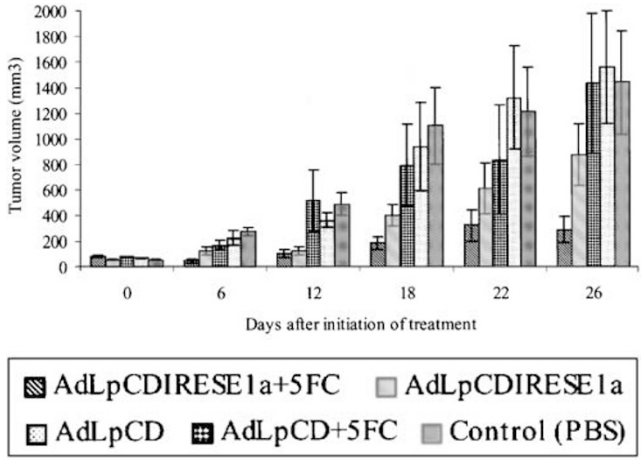



**Figure 5** Effect of intratumoral injection of tumor nodules with adenoviral vectors on tumor growth. The HTB-38 cells ($3 \times 10^6$) were injected s.c. into nude/nude mice. Following the growth of tumor nodules to around 50 $mm^3$, $10^8$ PFU of the AdLpCDIRESE1a and AdLpCD vectors were injected 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.

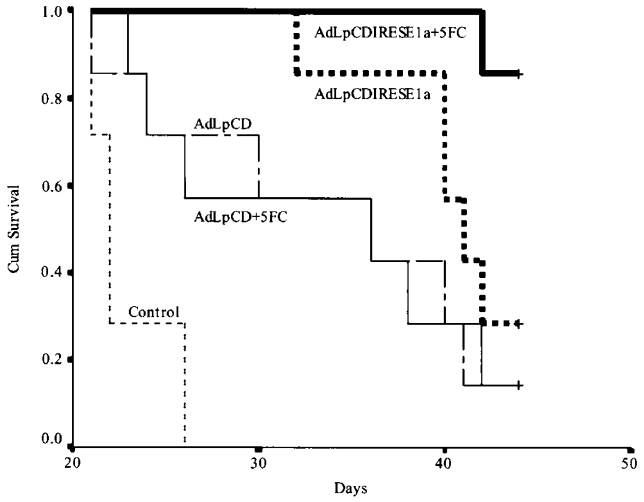



**Figure 6** Kaplan–Meier survival curves of nude mice with tumor nodules from HTB-38 colon cancer. The mice in all groups were followed until death or killing because of the large tumor size or at the end of 44 days of observation. At that time, most of the mice that were alive were killed because of 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* bystander 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 $\mu$M. The injection of the AdLpCDIRESE1a 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

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