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13. ABSTRACT *(Maximum 200 Words)*

The L-plastin promoter has been shown to promote expression of therapeutic and proliferation genes in adenoviral vectors which is tumor specific. In the last year, this grant has supported the study of replication incompetent adenoviral vectors with cytosine deaminase therapeutic genes regulated by the L-plastin promoter. These vectors have been shown in cell lines, primary explant cultures and animal models to be selectively toxic to ovarian and breast cancer cells. Adenoviral vectors with the E1A gene under control of the L-plastin promoter have also been constructed in this grant. These conditionally replication competent vectors have been shown in cell lines and primary explant models to be selectively toxic to breast and ovarian cancer cells. These data suggest that such vectors may be of value for new directions in treatment of breast cancer in advance disease patients. Work to be carried out in the next year include construction of adenoviral vectors in which both the cytosine deaminase and E1A genes are regulated by the L-plastin promoter, and the study of this new vector as well as the conditionally replication competent vectors in which the E1A gene is regulated by the L-plastin promoter in cell lines, primary explant cultures and animal models.

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5. Indroduction:

This proposal summarizes a new approach for the treatment of metastatic breast cancer. We have engineered a cold virus so that becomes specifically toxic to tumor cells but not to normal cells. This is based on the introduction a tumor specific transcriptional promoter 5' to the E1A gene of the virus which controls the ability of the virus to replicate itself within the cells of the body. This vector will not replicate within normal cells but will replicate within the tumor cells and therefore kill them. We have developed such a safety modified virus in which the tumor specific L-plastin promoter which 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, 5FC into a toxic drug, 5FU. Alternatively, the L-plastin promoter can be used to drive the expression of the viral E1A gene, which the adenovirus needs to replicate within mammalian cells. The data presented within this proposal shows that such vectors can replicate within breast cancer cells and other estrogen dependent carcinomas, such as ovarian cancer, while not being able to replicate in normal cells. The same transcription units driven by the L-plastin promoter are not expressed in primary cultures of normal tissue, but are expressed in primary cultures (monolayer and organ culture) of cancerous tissue. These vectors may therefore be developed into therapy for metastatic breast cancer which is less toxic than chemotherapy and which is more effect at controlling the progression of the disease.

6. Body:

Approved Statement of Work:

Specific Aim #1: Study of the Tumor Specific L-Plastin Promoter Cytosine Deaminase Transcription Unit is a Replication Incompetent Vector:

1.Tumor Selective Pattern of Expression of L-plastin Promoter Driven Genes in Adenoviral Vectors Tissues: We first introduced the LacZ gene driven by either the L-plastin or the CMV tumor non-specific promoters into a replication incompetent adenoviral vector. We had already showed (12) that the replication incompetent adenoviral vector carrying the LacZ reporter gene (which turn cells blue), controlled by the L-plastin promoter (Ad-Lp-LacZ), produces beta-galactosidase (detectable as blue

color in cells) expression in established breast cancer cell lines infected with these vectors, as shown in Figure 1A. These studies suggested that breast cancer tissue is infectable by the virus and that the regulatory environment of breast cancer cells supports transcriptional activation of genes driven by the L-plastin promoter.

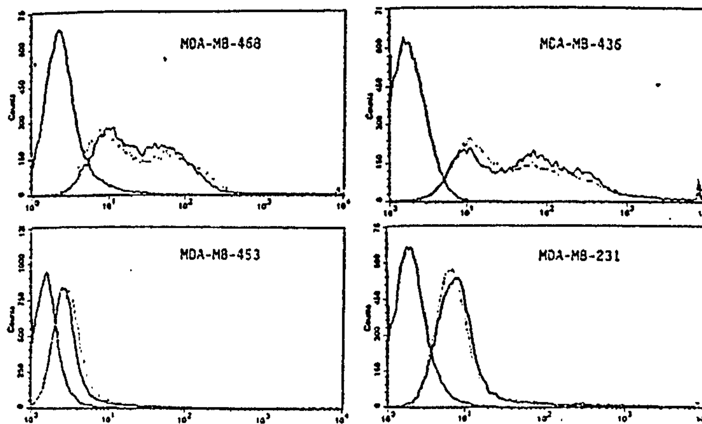
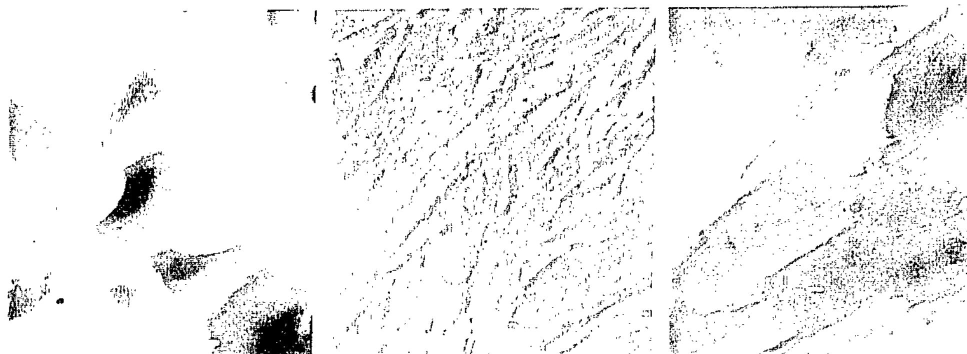


Figure 1A: Breast Cancer Cell Lines Infected with Ad-Lp-LacZ

We then tested whether infection of explant or primary cultures of cancer cells derived from the biopsy of an estrogen dependent tumor (ovarian cancer) would support the expression of the L-plastin promoter. The L-plastin driven LacZ adenoviral transcription was highly expressed in primary ovarian cancer monolayer explants (see Figure 2A). In contrast, the same Lp-Lac Z adenoviral transcription unit was not expressed seen in the minimal deviation CCD fibroblast cell line which resembles the normal peritoneal lining cells following exposure to the Ad-Lp-LacZ (see Figure 2B). The same vector turns CCD cells blue when exposed to an adenoviral vector carrying the LacZ gene driven by a tumor non specific CMV promoter (see Figure 2C), suggesting that the CCD cells are infectable by the adenoviral vector and capable of expressing LacZ when driven by a tumor non specific promoter



Panel 2A Panel 2B Panel 2C

Figure 2: Comparison of Primary Ovarian Cancer Cell Cultures and CCD Fibroblast Cultures Exposed to Ad-Lp-LacZ or Ad-CMV-LacZ adenoviral vectors.

2.Study of the Efficacy of Replication Incompetent Adenoviral Vectors Carrying a Chemotherapy Sensitization Gene: We then assembled replication incompetent adenoviral vectors in which the L-plastin (tumor specific) or the CMV (tumor non specific) promoters were driving the cytosine deaminase gene (CD, a bacterial gene which catalyzes the conversion of a non toxic prodrug (5 Fluorocytosine: 5FC) into a toxic chemotherapy agent (5 Fluorouracil: 5FU). Although when 5FU is used clinically, it kills primarily dividing cells, the levels of 5FU substitution of mRNA in cells infected with an adenovirus carrying the CD gene under the control of the CMV promoter is so

Panel A Panel B

Figure 4: Organ Cultures of Normal Ovarian Tissue Exposed to the Ad-CMV-LacZ Replication Incompetent Adenoviral Vectors (Panel A) or the Ad-Lp-LacZ (Panel B). The normal ovarian cells show a band of blue color at the edge of the organ culture when exposed to the adenoviral vector carrying the CMV driven LacZ transcription unit but no blue color when the same normal ovarian cell organ culture is exposed to the adenoviral vector carrying the LacZ gene driven by the tumor specific L-plastin promoter.

high, that non dividing cells will die due to interruption of mRNA processing and protein synthesis. Adenoviral vectors carrying a CMV driven CD transcription unit have already been used clinically. We decided to introduce the CD gene downstream of the L-plastin promoter to see if we could create a vector which sensitized tumor cells to the toxic effects of 5FC more than normal cells. We first tested whether the breast cancer cell can be sensitized to the toxic effects of the 5FC precursor drug by exposure to the replication incompetent adenoviral vector which carries the CD gene regulated by the CMV promoter (Ad-CMV-CD). In a study published from our laboratory (12), we reported that the MCF-7 breast cancer cell line colony forming units were reduced by more than one million fold by exposure to the Ad-CMV-CD vector.

We then compared the effects of the Ad-Lp-CD (tumor specific promoter) with that of the Ad-CMV-CD (tumor non specific promoter) in the Ovcar-5 ovarian cancer cell line

We had also reported (see Appendix A) that primary explant cultures of metastatic, and ascitic ovarian cancer cells, obtained as biopsies from patients (Panels A and B) expressed higher levels of the LacZ gene when exposed to the Ad-Lp-LacZ Vector (see Figure 3A) than did primary explants of mesothelial cells, which are the normal cells which line the peritoneal cavity (Figure 3E). The mesothelial cells turned blue following exposure to an adenoviral vector carrying the LacZ gene driven by a tumor non specific promoter (CMV), as shown in Figure 3, Panel F. This suggested that the absence of blue color in the mesothelial cells exposed to the L-plastin LacZ vector is not due to lack of infection, but failure of the cellular environment to activate the L-plastin promoter.

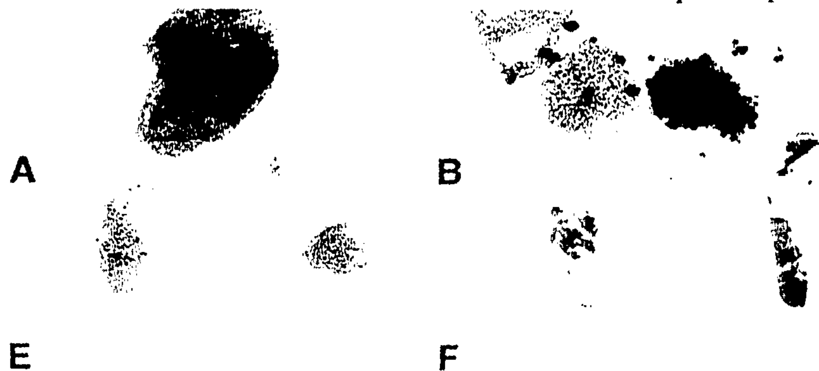


Figure 3: Comparison of Explants of Ovarian Cancer (upper Panels A and B) vs Primary Explants of Mesothelial Cells (lower Panels E and F) Exposed to the Ad-Lp-LacZ vector (Panels A and E-left hand side) versus the Ad-CMV-LacZ(Panels B and F-right hand side).

A paper has been published on the work presented in Figures 1-3 (Chung I, Schwartz PE, Crystal, RG, Pizzorno G, Leavitt J, and Deisseroth, AB, "Use of L-plastin promoter to develop an adenoviral system that confers transgene expression in ovarian cnacer cells but not in normal mesothelian cells", Cancer Gene Therapy 6: 99-106, 1999. This is presented in Section 11 (Appendices) as Appendix A.

We also carried out in collaboration with Dr. Eva Sapi of diagnostic radiology experiments designed to test whether primary cultures of normal epithelial tissue would display the suppression of the L-plastin promoter which was seen in the cell lines studies above (see Appendix B). Since Dr. Sapi was studying organ cultures of epithelial cells from normal ovary, another estrogen dependent tissue like breast, we provided Dr. Sapi with two replication incompetent adenoviral vectors, one in which the Lac Z gene was driven by the CMV tumor non-specific promoter, and another in which the Lac Z gene was driven by the L-plastin promoter. The data from this experiment is shown below in Figure 4.

and in the minimal deviation fibroblast cell lines. In order to carry out this analysis, we exposed each cell line to each vector under conditions which would result in 100% of the cells being infected. We then made artificial mixtures of the infected cells with uninfected cells to generate mixtures of infected and uninfected cells which ranged from 0% to 100% (see the abscissa to Figure 5). We then exposed the cells to 500 micromolar concentration of the non toxic prodrug 5FC and measured 5 days later the surviving cells. As shown in Figure 5, the ratio of cell kill following exposure to the Ad-CMV-CD/Ad-Lp-CD vectors at a MOI of 160 is 1/1 in the case of the Ovcar-5 ovarian cancer cell line whereas the ratio of cell kill in the CCD minimum deviation fibroblast cell line is 4/1 following exposure to the Ad-CMV-CD/Ad-Lp-CD vectors. Thus, the differential expression in tumor versus non cancerous cells seen with the L-plastin promoter regulated LacZ gene is also seen with the L-plastin regulated CD gene.

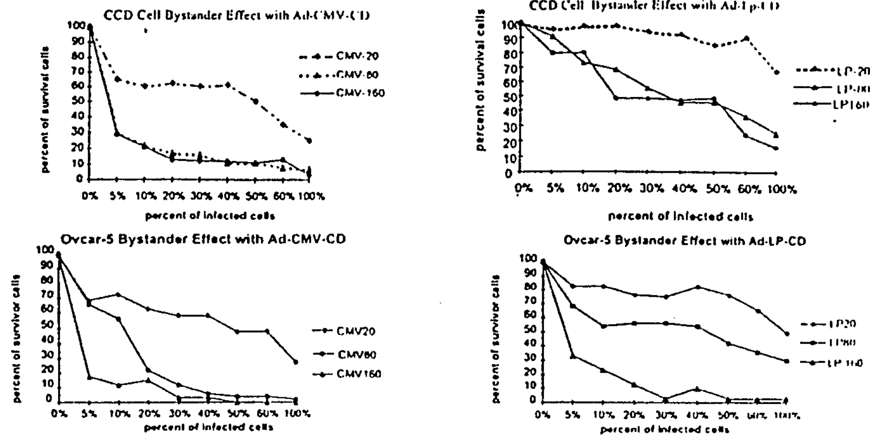


Figure 5: Comparison of Cytoxicity with the Ad-CMV-CD (left panels) vs Ad-Lp-CD (right panels) Vectors in the Ovcar-5 Ovarian Cancer Cell line (lower panels) vs the Minimal Deviation CCD Fibroblast Cell Line (upper panels).

<u>3.Animal Model Studies of Replication Incompetent Adenoviral Vectors Carrying Therapeutic Genes Driven by the L-plastin Promoter:</u> In order to test the in vivo efficacy of the Ad-Lp-CD replication incompetent vector system, the Skov 3 or the Ovcar 5 ovarian cancer cell lines were exposed to either the Ad-Lp-CD vector or a vector without a CD gene at a MOI of 100 by incubating the cells in the vector for 90 minutes. Then we injected one million per mouse of either Ovcar 5 or Skov 3 ovarian carcinoma cell lines into 10 or 5 mice respectively. One day after the injection of the tumor cells, we initiated daily intraperitoneal injections of each of the animals with sufficient 5FC to generate peak intraperitoneal 5FC concentrations of 500 micromolar. We carried on the daily intraperitoneal injections for 10 days after the tumor injection. We then sacrificed the majority of animals at day 21 and a few were left to be sacrificed on day 50 after injection. As shown in Table I below, all of the animals injected with ovarian cancer cells exposed to the Ad-Lp-CD vector were free of detectable tumor cells, either at the gross or

histochemical levels. In contrast, as shown in Table I, all 10 of the Ovcar 5 and all 5 of the Skov 3 animals injected with ovarian cancer cells infected with the backbone virus had detectable tumor cells. We also examined the peritoneal surface on a gross level at autopsy for signs of inflammation in the animals injected with the tumor cells infected with the Ad-Lp-CD vector and injected with 5FC for signs of inflammation. The peritoneal surface was totally normal in these animals both at the gross and microscopic level. This data shows that the toxic effect of the Ad-Lp-CD system is specific for the tumor cells, and that there is no "bystander effect" damage on the normal cells of the peritoneum.

Table I: In Vivo Study of Chemotherapy Sensitization Effect of the Replication Incompetent Adenoviral Vector Carrying the L-plastin Regulated Cytosine Deaminase Transcription Unit

	Animals Showing Tumor Nodules in Peritoneal Cavity	
	Non-Infected with Ad-Lp-CD	Infected with Ad-Lp-CD
Ovcar 5	10/10 (100%)	0/10 (0%)
Skov3	5/5(100%)	0/5 (0%)

A paper has been completed on the work presented in Figures 4-5 and Table I (Peng, XY, Rutherford T, Won JH, Pizzorno, G, Sapi E, Kacinski B, Crystal, R, Schwartz P, and Deisseroth, A. The use of the L-plastin promoter for adenoviral mediated tumor-specific gene expression in ovarian and bladder cancer cell lines", under revision for publication at this time. The manuscript is presented in Section 11 (Appendices), Appendix B.

Thus, the hypothesis outlined in the Task outlined in Specific Aim #1 in the original application: that the L-plastin promoter could confer gene expression in the replication incompetent adenoviral vector which is specific for breast and ovarian cancer cells has been sustained.

Specific Aim #2: Assembly of the Conditionally Replication Competent Adenoviral Vector with the Tumor Specific L-Plastin Promoter Cytosine Deaminase Transcription Unit:

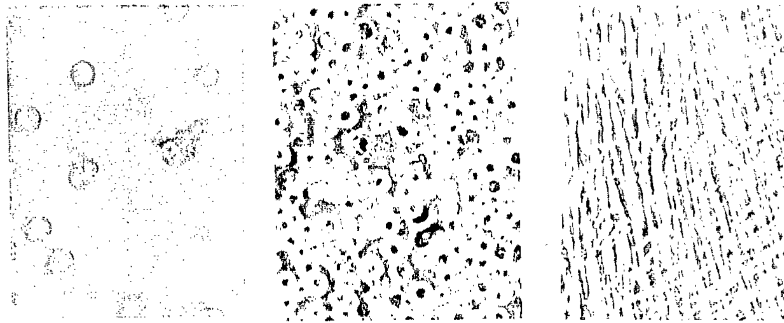
We have proposed to construct adenoviral vectors which will specifically replicate within breast and ovarian cancer cells and thereby lead to the lysis of these cancer cells without harming any normal population of cells. We first carried out experiments with reporter

genes regulated by the L-plastin to show that the expression of L-plastin driven genes occurs in breast and ovarian cancer cells but not in non malignant cells.

4. Study of Cell Lines and Primary Cultures of Tumor Cells Exposed to Conditionally Replication Competent Adenoviral Vector in which the L-plastin Promoter Controls the Expression of the E1 Viral Replication Gene: Now that we had a molecular switch to turn genes on in the tumor but not in the normal cells, we then used the method of He et al (19) to assemble conditionally replication competent adenoviral vectors with the tumor specific L-plastin promoter (Ad^{RC}-Lp-E1A) or the CMV promoter (Ad^{RC}-CMV-E1A) controlling the adenoviral E1A gene which the virus needs to replicate within cancer cells. These vectors are designed to be directly toxic to the cancer cells without damaging the normal cells.

We first exposed established cell lines derived from carcinomas of the breast and ovary to the Ad^{RC}-Lp-E1A vectors. As shown in Panel A of Figure 6, the vector carrying the E1A gene driven by the L-plastin promoter completely lysed the MDA-468 breast cancer cell line (all cells detached or rounded 7 days after infection at a MOI of 100), and as shown in Panel B of Figure 6, lysed the Ovcar-5 ovarian cancer cell line (all cells rounded up in 7 days following infection at a MOI of 10). In contrast, there was no detectable rounding up or lysis of cells in the CCD minimal deviation cell at 7 days following infection at a MOI of 10. We had shown that the CCD was infectable with the Adenoviral vector, but that it did not support the activation of expression of Lp-driven genes (see Figure 2 above, panels B and C). This data suggested that the L-plastin promoter could be used to create vectors which were directly lytic to breast and ovarian cancer cells but not normal cells.

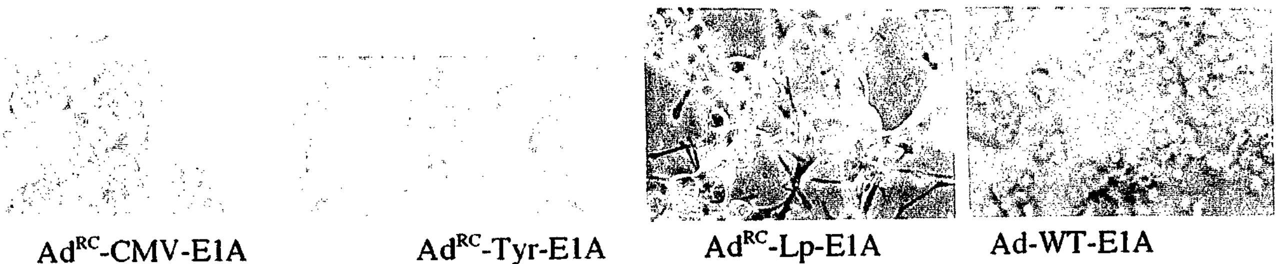
In order to determine the potential specificity of such conditionally replication competent vectors, we introduced the tyrosinase promoter (specific for melanoma cells) 5' to the E1A gene in the adenoviral vector (Ad^{RC}-Tyr-E1A). This promoter was also supported by an enhancer found to be necessary for optimal performance of the Tyrosinase promoter. We then compared the following cell lines :CCD- (does not support Lp driven genes), TF (melanoma cell line: should support Tyrosinase promoter driven genes), and MDA 468 (a breast cancer cell line which supports L-plastin promoter driven genes), to the following vectors: wild type adenoviral vector (AdWT), Ad^{RC}-CMV-E1A, Ad^{RC}-Tyr-E1A, and Ad^{RC}-Lp-E1A. As shown by the data presented in Figure 7, the TF melanoma



Panel A (MDA 468) Panel B (Ovcar-5) Panel C (CCD)

Figure 6: Comparison of the Cytotoxicity in MDA 468, Ovcar-5 and CCD cell lines Following Exposure to Ad^{RC}-Lp-E1A Replication Competent Adenoviral Vector

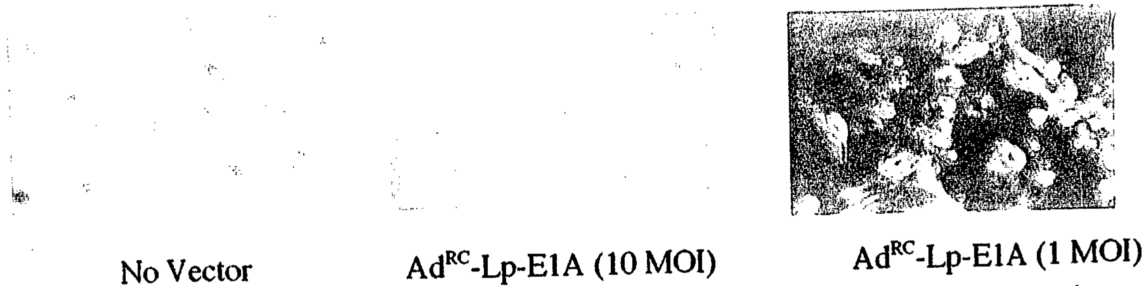
cell line is totally lysed by the Ad^{RC}-Tyr-E1A, the Ad^{RC}-CMV-E1A and the Ad-WT-E1A, whereas the L-plastin promoter does not drive the expression of the E1A gene sufficiently in the melanoma cell line to generate total lysis of the cells under the conditions of the experiment (MOI 10 for 9 days).



Ad^{RC}-CMV-E1A Ad^{RC}-Tyr-E1A Ad^{RC}-Lp-E1A Ad-WT-E1A

Figure 7: Analysis of the Cytotoxicity of the Above Vectors in the TF Melanoma Cell Line.

As shown in Figure 8, we tested the extent of lysis of a primary culture of ovarian carcinoma cell plated in monolayer following exposure to theAd^{RC}-Lp-E1A vector. The results indicate that the L-plastin promoter drives the expression of the E1A gene sufficiently in the primary ovarian carcinoma cells at a MOI of 0.1 to lyse most cells.



No Vector   Ad^{RC}-Lp-E1A (10 MOI)   Ad^{RC}-Lp-E1A (1 MOI)

Figure 8: Study of the Sensitivity of Primary Cultures of Ovarian Cancer Cells to the Ad^{RC}-Lp-E1A cultures.

Thus, the original hypothesis of theTask outlined in Specific Aim #2 in the original application: that the L-plastin promoter could be used to confer tumor specific conditional replication competency on adenoviral vectors so as to render those vector specifically toxic to ovarian and breast cancer cell lines and primary tissue, has been sustained by this data.

Specific Aim #3:Study of the Tumor Specific L-Plastin Promoter Cytosine Deaminase Transcripiton Unit in a Replication Competent Vector:

This Task has not yet been completed.

7. Key Research Accomplishments:

The following are the key accomplishments of this research:

a. Development of replication incompetent adenoviral vectors in which the therapeutic transgene, Cytosine Demaminase, is under the control of the L-plastin promoter, thereby making the vectors selectively sensitizing of ovarian and breast cancer to the prodrug, 5 fluorocytosine. These vectors are not toxic to normal cells.
b. Develop of replication competent vectors in which the viral E1A gene has been placed under the control of the L-plastin promoter, thus making these vectors selectively toxic to the breast and ovarian cancer cells. These vectors are not toxic to normal tissue.

8. Reportable Outcomes:

a. Publication a manuscript (Chung, I, Schwartz, PE, Crystal RG, Pizzorno, G, Leavitt, J, and Deisseorth, AB, Use of L-plastin promtoer to develop an adenoviral system that confers transgene expression in ovarian cancer cells but not kin normal mesothelial cells, Cancer Gene Therapy 6: 99-106, 1999). See Section 11-Appendices, for Appendix A.

b. Completion and Submission of a manuscript for publication (Peng, XY, Rutherford T, Won JH, Pizzorno G, Sapi E, Kacinski, B, Crystal, R, Schwartz, P, and Deisseroth A. The use of the L-plastin promoter for adenoviral mediated tumor-specific gene expression in ovarian and bladder cancer cell lines. Submitted to Cancer Research). See Section 11-Appendices, for Appendix B.

c. Application for a patent based on the use of the L-plastin promoter for creating tumor specific transcription units and conditionally replication competent vectors which are specifically cytolytic for tumor cells.

d.Development of adenoviral vectors which are conditionally replication competent in and cytolytic for breast and ovarian cancer cells.

9. Conclusions:

The results presented in this progress report shows that the two hypotheses presented in Specific Aims #1-2 of the Task in the approved grant application are achievable. These data show that it is possible to create replication incompetent vectors which display tumor specific expression of therapeutic transgenes. This data has been developed in cell lines, primary explants of normal and neoplastic tissue, and in human tumor cell line xenografts in nude mice. In addition, the results show that it is possible to create vectors which are replication competent only in tumor cells and thereby are directly cytolytic for tumor cells but not in normal cells. This data has been developed in primary explants of human tumor cells, and in cell lines.

The future work will address the study of the conditionally replication competent vectors in a human tumor xenograft model in nude and SCID mice, and in the development of conditionally replication competent adenoviral vectors in which the therapeutic genes are

expressed in a tumor specific manner. This goal was outlined in the original application as an approved Task in Specific Aim #3.

10. References:

1. Chung I, Schwartz PE, Crystal RG, Pizzorno G, Leavitt J, and Deisseorth, AB. 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 Therapy 6: 99-106, 1999.
2. Peng XY, Rutherford T, Won JH, Pizzorno G, Sapi E, Kacinski B, Crystal R, Schwartz P, and Deisseroth A. The use of the L-plastin promoter for adenoviral mediated tumor-specific gene expression in ovarian and bladder cancer cell lines. Submitted, 2000.

11. Appendics:

Appendix A: Chung I, Schwartz PE, Crystal RG, Pizzorno G, Leavitt J, and Deisseroth, AB. 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 Therapy 6: 99-106, 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

Injae Chung,[1] Peter E. Schwartz,[1] Ronald G. Crystal,[2] Giuseppe Pizzorno,[1] John Leavitt,[3] and Albert B. Deisseroth[1]

[1]Yale University School of Medicine, New Haven, Connecticut 06520; [2]The New York Hospital, Cornell Medical Center, New York, New York 10021; and [3]Palo Alto Medical Foundation Research Institute, Palo Alto, California 94301.

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

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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.

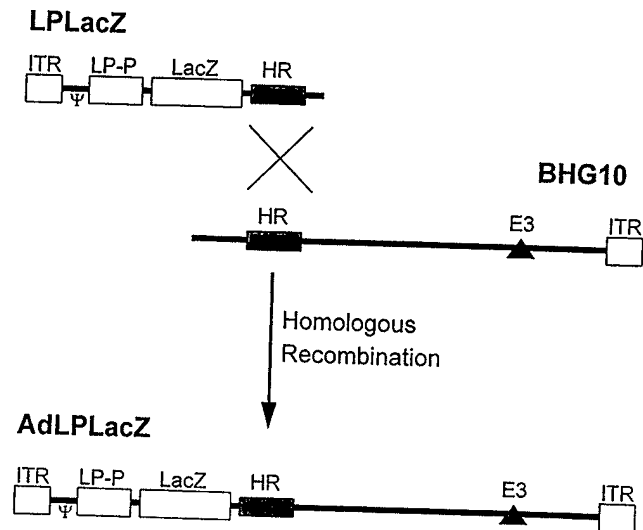

Figure 1. Assembly of AdLPLacZ adenoviral vector by the homologous recombination method. LPLacZ is a shuttle vector that contains an adenoviral inverted terminal repeat (ITR) and packaging signal (Ψ), the *LacZ* gene driven by the LP-P, and some adenoviral vector sequences that are homologous in sequence (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.

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 *Sca*I 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 *Sca*I 2.4-kb fragment to the *Hin*cII site of pBluescript II SK$^-$ (Stratagene, La Jolla, Calif), to produce pLPSK (see Fig 1). The *Spe*I/*Xho*I-digested 2.4-kb fragment of pLPSK was then ligated to the *Spe*I/*Xho*I-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.

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-

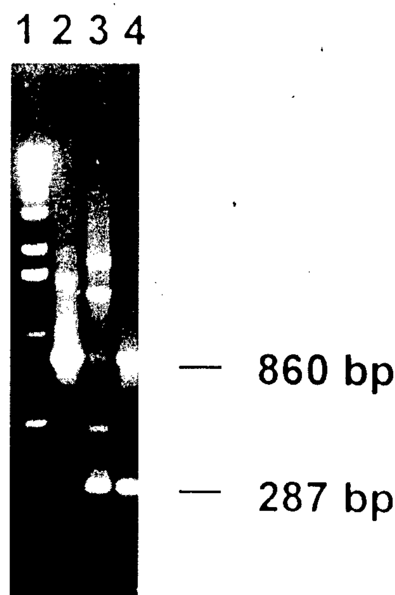

Figure 2. PCR analysis of recombinant adenoviral vector with LP-P/*LacZ* gene. DNA was isolated from plaques resulting from calcium phosphate transfection of the AdLPLacZ shuttle vector and the pBHG10 adenoviral vector with a deletion in the E1 gene region. The homologous recombination event would take 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

	FACS-gal analysis	
Cell lines	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 AdCMVLacZ 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.

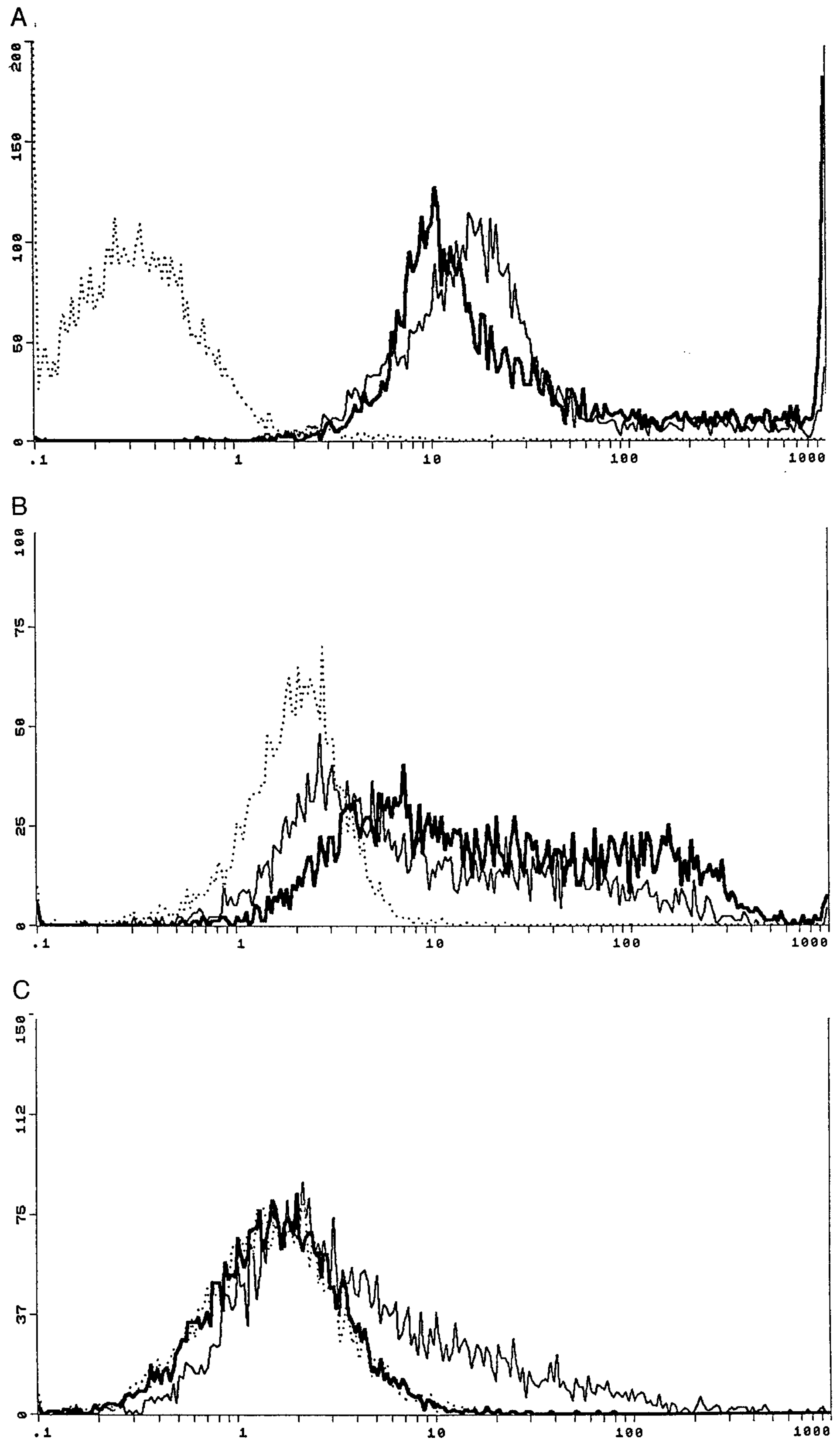

Figure 3. FACS-gal analysis of the cell lines exposed to AdLPLacZ and AdCMVLacZ adenoviral vectors. Cell lines derived from patients with: **A**, breast cancer (MDA-MD-468); **B**, ovarian cancer (SKOV-3); **C** and **D**, skin fibroblasts (CCD-862SK and CCD-944SK); **E**, hematopoietic leukemia cells (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.

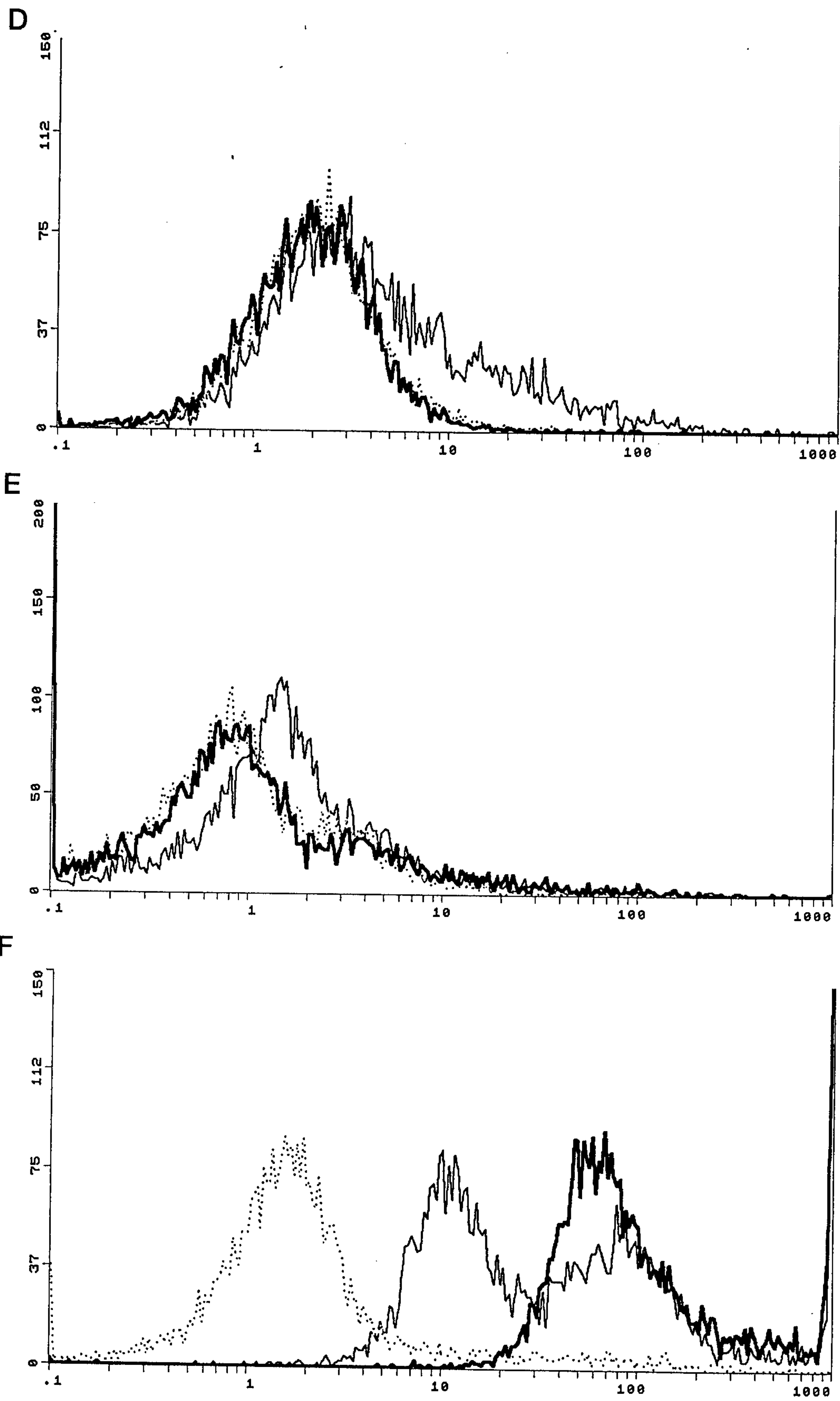

Figure 3. Continued

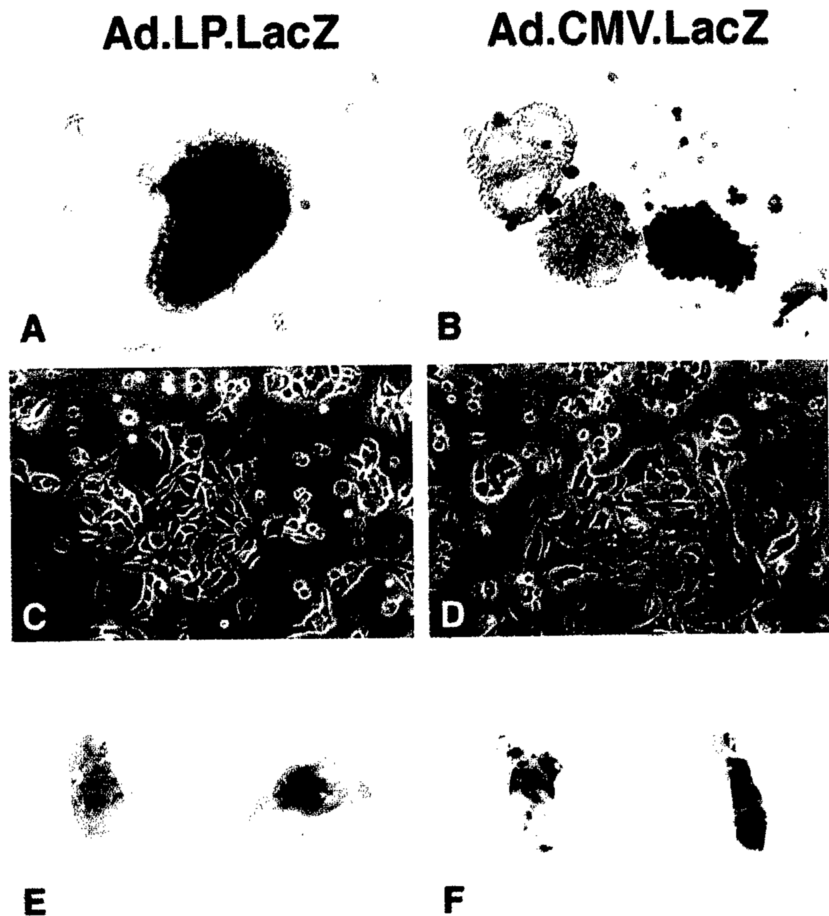

Figure 4. X-Gal staining studies of normal mesothelial cells and malignant ascites from an ovarian cancer patient. Fresh samples from patients were exposed to 0.5 mL of medium containing 150 million plaque-forming units of the adenoviral vector (except for **E** and **F**, in which 50 million plaque-forming 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 AdCMV-LacZ 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 AdL-PLacZ (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 αVβ3 and αVβ5 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.

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11. Appendices.

Appendix B: Peng XY, Rutherford T, Won JH, Pizzorno G, Sapi E, Kacinski B, Crystal R, Schwartz P, and Deisseroth A. The use of the L-plastin promoter for adenoviral mediated tumor specific gene expression in ovarian and bladder cancer cell lines. Submitted 2000.

The Use of the L- Plastin Promoter for Adenoviral Mediated Tumor-Specific Gene Expression in Ovarian and Bladder Cancer Cell Lines

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Abstract

A 2.4Kb truncated L-plastin promoter was inserted either in 5' to the LacZ gene (Ad-Lp-LacZ) or 5' to the cytosine deaminase gene (Ad-Lp-CD) in a replication incompetent adenoviral vector backbone. Infection experiments with these vectors showed that the L-plastin promoter driven transcriptional units were expressed at much higher levels in ovarian cancer cells from patients and in ovarian or bladder cancer cell lines than they were in normal mesothelial cells from surgical specimens or in the CCD minimal deviation fibroblast cell line. Control experiments showed that this difference was not due to lack of infectivity of the normal peritoneal cells or the minimal deviation cell line, since these cells showed expression of the LacZ reporter gene when exposed to the replication incompetent adenoviral vector carrying the CMV driven LacZ gene (Ad-CMV-LacZ). The Nutu-19, Ovcar-5, and Skov3 ovarian cancer cell lines exposed to the Ad-Lp-CD adenoviral vector were much more sensitive to the prodrug 5-Fluorocytosine (5FC) than was the CCD minimal deviation fibroblast cell line following exposure to the same vector. Finally, a mouse xenograft model was used to show that the Ad-Lp-CD vector/5FC system could prevent engraftment of ovarian cancer cells in SCID mice. Thus, these results suggest that an adenoviral vector carrying the cytosine deaminase gene controlled by the truncated L-plastin promoter (Ad-Lp-CD) may be of potential value for the intraperitoneal therapy of ovarian cancer.

Introduction

Adenoviral vectors are currently the most frequently used vector 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 non dividing cells . A possible disadvantage of this vector is that the broad host range of this virus also results in the infection of both the intended tumor cells as well as of the surrounding normal tissue. This limits the utility of these vectors, especially when the vector gene products are designed to sensitize tumor cells to chemotherapy or radiation therapy, due to unwanted toxicity for normal cells (Descamp et al., 1996; Bramson et al., 1995; Zhang et al., 1994).

One way to circumvent this limitation would be to employ a tissue-specific transcriptional promoter active only in the target tumor cells. Our laboratory has constructed adenoviral vectors in which the L-plastin promotor is employed 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 John Leavitt and his collegues(Leavitt et al., 1994; Lin et al., 1997; Park et al., 1994). The only normal cell in which this protein is detectable is the mature leukocyte. This protein has been demonstrated to be present in greater than 90% of epithelial neoplastic cells, and is not found in normal epithelial cells. Therefore, the L-plastin promotor is of potential utility in cancer gene therapy since it can be used to drive the expression of heterologous genes in a tumor specific manner in the context of recombinant adenoviral vectors. Injae Chung in our laboratory had previously reported 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(Chung et al., 1999).

We now are reporting the results of experiments based on replication-deficient adenoviral vectors which contain either a LacZ reporter gene or a cytosine deaminase (CD) therapeutic transcriptional unit regulated by a 2.4 Kb fragment of the L-plastin promoter in bladder and ovarian cancer cell lines, in normal and neoplastic ovarian primary tissue in organ culture, and in ovarian cancer cell lines in a nude mouse animal model. Cytosine deaminase (CD) is a bacterial gene which converts 5-Fluorocytosine (5FC), which is non toxic to cell lines and primary cells, into 5-Fluorouracil (5FU), a cytotoxic compound. The levels of phosphorylated 5FU generated within CD positive cells are sufficiently high that even non dividing cells die due to disruption of mRNA processing and protein synthesis.

The results of these experiments have shown that:

1. The level of L-plastin promoter driven LacZ heterologous reporter gene expression is lower in a minimal deviation fibroblast cell line (CCD) when compared to a collection of established epithelial tumor cell lines derived from ovarian cancer, and bladder cancer.

2. The L-plastin promoter activates the LacZ and cytosine deaminase transcription units to a higher level in ovarian cancer cells than in primary monolayer and organ cultures of normal ovarian tissue, or of normal peritoneal tissue.

3. The replication incompetent adenoviral vector carrying the cytosine deaminase transcription unit driven by the L-plastin promoter selectively kills ovarian and bladder

cancer cell lines in vitro and in SCID mice exposed to 5FC. These results suggest that adenoviral vectors carrrying the cytosine deaminase transcription unit driven by the L-plastin promoter may be of utility in the intraperitoneal treatment of metastatic ovarian cancer.

Material and Methods

Cells and Cell Culture: Human bladder carcinoma cell lines (J82, EJ) were obtained from Dr. Richard Cote of the USC. The following cell lines were obtained from American Type Culture Collection (ATCC): the CCD minimal deviation human fibroblast cell line, the 293 transformed human kidney cell line, and the Skvo3 ovarian cancer cell line. The Hey cystadenocarcinoma papillary ovarian carcinoma cell line was obtained from Dr. Eva Sapi. The Nutu-19 rat ovary adenocarcinoma cell line, and the Ovcar-5 human epithelial ovary carcinoma cell line were obtained from Dr. Thomas C. Hamilton of the Fox Chase Cancer Center, Philadelphia, PA.

Chemicals and Reagents: 5-FC, 5FU, fluorescein di-β-D-galactopyranoside (FDG), X-gal (4-bromo-5-chloro-3-indoyl-β-D-galactopyrosanide) were purchased from Sigma Chemical Co.. 6-^{3}(H)5-Fluorocytosine(4.1Ci/mmol) and 6-^{3}(H)5-Fluorouracil were purchased from Noravek Biochemicals Inc. of Brea, CA. Monoclonal antibodies to αv β3 (LM609) and αvβ5(P1F6) integrins were purchased from Chemicon International. A monoclonal antibody to the Coxsackie virus receptor (CAR), which binds the adenoviral fibrillar protein, was obtained from Dr. R.W. Finberg of the Dana-Farber Cancer Institute, Harvard Medical School, Boston, Mass.

Construction of Replication Incompetent Recombinant Adenoviral Vectors: The Ad-CMV-CD vector (Hirschowitz et al, 1995), which contained the cytosine deaminase gene controlled by a cytomegalovirus (CMV) promoter in place of the adenoviral E1A and E1b gene, was obtained from the laboratory of Dr. Ron Crystal of the Cornell Medical School. 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 (Garcia-Sanchez et al., 1998). Injae Chung of our laboratory truncated the 5Kb L-plastin promoter to a 2.4Kb fragment, which extended from nucleotide -2265 of the 5í region of the L-plastin promoter to +18bp from the transcription initiation site of the L-plastin gene. 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(Graham et al., 1991; Neer et al., 1996).

Analysis of Cellular Receptors on Tumor Cells which Participate in Vector Uptake: Mouse monoclonal antibodies to the αvβ3 [LM609] integrin, the αvβ5 [P1F6] integrin, and to the CAR receptors were used to detect the density of the human αvβ3, αvβ5 and CAR receptors on the test cells. The FACS Star Flow Cytometer (Becton Dickinson) in the Yale Cancer Center was used to determine the percentage of cells positive for each receptor.

β-galactosidase Activity Assay:

1. X-gal staining: The viable cell number was counted by Trypan Blue exclusion. The cells were fixed with ice-cold 2% paraformaldehyde/0.2% glutaraldehyde for 10 minutes. The level of β-gal expression cells was then assessed by staining the cultures with 4-bromo-5-chloro-3-indoyl-β-D-galactopyrosanede (X-Gal) and potassium-ferrcyanide/ferrocyanide solution essentially as described previously(Couffinhal et al., 1997; Lawrence et al., 1998). The average number of β-gal expressing (blue) cells per well was determined by counting 5 separate microscopic high-power fields and then the average of the 5 fields determined.

2.Fluorescein di-β-d-galactopyranoside (FDG) Flow Cytometry Analysis of LacZ Report Gene Expression in Cells Exposed to the Ad-CMV-LacZ and Ad-Lp-LacZ Vectors : $3x10^5$ cells were exposed for 90 minutes to the Ad-CMV-LacZ or Ad-Lp-LacZ vectors at different MOI in DMEM medium supplemented with 2% serum and chloroquine, were subjected to FACS-gal analysis as described by Roederer et al (Roederer et al., 1991). The cells were then exposed to 50ul of 2mM fluorescein di-β-d-galactopyranoside (FDG) for 60 seconds, following which 1ml of ice-cold quench solution (staining solution with propidium iodide) was added and the cells were then further incubated on ice for 1hour. After this, phenylethyl β-D-thiogalactopyranoside (PETG) was added to stop the reaction. Then, the cells were subjected to FACS analysis.

The Effect of 5-FU Released from Cytosine Deaminase 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 (20MOI, 80MOI, 160MOI) using the Ad-CMV-CD or Ad-Lp-CD vectors. The infected cells and non-infected cells were mixed in varying ratios to generate 0, 5%, 10%, 20%, 30%, 40%, 50%, 60%, and 100% of infected cells. Cells then were seeded in triplicate in six well tissue culture plates and incubated for 24 hours. This was followed by incubation with 500umol/L 5FC for 6 days. The number of surviving cells was determined using Trypan Blue exclusion(Kianmanesh et al., 1997).

Comparison of the 5-FU Sensitivity (IC50) of Ovarian Cancer and Bladder Cell Lines with CCD(Minimal Deviation Fibroblast Cell Lines): The concentrations of 5FU used for the cytotoxicity test (IC50) were 100, 50, 10, 1, and 0.5uM. After 96 hours, the cells were removed with trypsin-EDTA and cell number calculated using the Coulter Counter ZM (Hialeah, FI).

The Toxicity of Adenoviral Vectors: 2×10^5 cells were infected with the Ad-CMV-LacZ , Ad-Lp-LacZ, Ad-CMV-CD, or Ad-Lp-CD vectors at a MOI of 0, 5, 20, 40, 80 and 160 for 90 minutes, and then seeded in six well plates in duplicate. 24 hours later, 0.5mM 5FC was added to each well, and then the cells were incubated for 5 days. The cells were then trypsinized and the surviving cells were counted using Trypan Blue exclusion. 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 Primary Cell Monolayer 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 with the Ad-CMV-CD and Ad-Lp-CD vectors, the cells were grown in T25 flasks until 80% confluency. The cells were then washed in PBS and then exposed to vector directly in the flasks containing DMEM supplemented with 2% NBCS for 90 minutes. The cells were then incubated for 5 days at 500umol/L 5FC concentration, and then the cell viability was determined by light microscopic examination.

Vector Studies on Primary Cell Organ Culture of Ovarian Cancer and Normal Ovarian Tissue: Each specimen was then cut into pieces of approximately 1-2mm^3, and immersed in 4ml of DMEM/Ham's F12 medium which was supplemented with 10% charcoal-stripped serum(Sapi et al., 1998). Cultures were incubated at 37^{0}C in 6 well plates on a shaking platform for 24-48 hours, after which, the tissues were exposed to the Ad-CMV-LacZ , Ad-LP-lacZ, Ad-CMV-CD or Ad-LP-CD viral vectors for 90 minutes in serum free medium. The tissues were then washed with PBS. The Ad-CMV-CD and Ad-LP-CD viral vectors containing wells were then incubated with 0.5 mM 5-FC at 48-72 hours of incubation. X-Gal staining was used to measure the Ad-CMV-LacZ and Ad-LP-LacZ expression on slides. The AD-LP-CD and Ad-

CMV-CD infected tissues were also fixed in O.C.T. and stained with hematoxylin/eosin.

Vector Studies in SCID Mice: Ovcar-5 tumor cell lines were infected in vitro at 100 MOI with either the Ad-LP-LacZ or Ad-Lp-CD viral vectors for 60 minutes, then washed with PBS, and then resuspended in PBS (4×10^7cells/1ml PBS). Ten 6-8 week old female SCID mice (25-28 grams in weight), which were purchased from Cox, Inc., Cambridge, MA, were injected intraperitoneally with 40 million Ovcar-5 ovarian carcinoma cells previously infected at 100 MOI with the Ad-Lp-LacZ vector (Molpus et al., 1996). An additional ten 6-8 week old 26-28 gram mice were injected intraperitoneally with Ad-LP-CD infected cells. From the second day, all of the 20 mice were injected once a day with 5-FC in 500 mg/kg intraperitoneally for 10 days. 3 weeks after tumor cell injection, the 10 Ad-LP-LacZ injected mice and 7 of the Ad-LP-CD injected mice were sacrificed and autopsied. At the 50th day, another 3 Ad-LP-CD injected mice were sacrificed and autopsied. 5 female Swiss nude mice were injected with 40 million Skov3 cells previously infected in vitro with the Ad-Lp-CD vector at 80 MOI. Another 5 mice were injected Skov3 cells infected at 80 MOI with the Ad-LP-LacZ vectors (4×10^7cell/ml PBS per mouse). Then, 500mg/kg of 5FC was injected intraperitoneally daily for 10 days into all of the 10 mice. 3 weeks later, the mice were sacrificed and autopsied.

Results

Correlation of Expression of the LacZ Gene in Adenoviral Vectors (Ad-CMV-LacZ) Exposed Cells and Levels of CAR/Integrin Receptor Expression in Ovarian/Bladder Cancer Cell Lines and Minimal Deviation Fibroblast Cell Lines: The integrin $\alpha v\beta 5$ receptor, and the Coxsackie B/Adenovirus Receptor (CAR) have been reported by others to play an important role in adenovirus infection (Bergelson et al., 1997,1998; Roelvink et al., 1998, Wickham et al., 1993,1994). Differences in the integrin receptor and CAR expression levels and the functional state of these receptors may explain differences in the expression of LacZ genes in cells exposed to the Ad-CMV-LacZ vectors in different cell lines. We therefore studied the level of expression of the integrin and CAR receptors in the Nutu-19, Ovcar-5, Skov3, and Hey ovarian cancer cell lines, in the EJ, and J82 bladder cancer cell lines, and in the CDD minimal deviation cancer cell line (Table1). The percentage of cells positive for the Lac Z expression was greater than 60% in all cases in which the positivity for CAR was greater than 29%, whereas the percentage of cells positive for Lac Z expression was less than 10% or less in cell lines in which the CAR was undetectable (see Table 1). Very low levels of LacZ expression were observed in the Hey human ovarian cancer cell line in which the CAR was undetectable.

The low level of CAR in NuTu-19 seen in Table 1 may be an artifact due to the non reactivity of the antibody to the human CAR with the rat CAR. The rest of the cell lines which were infectable by the Ad-CMV-LacZ, as judged by the high level LacZ

expression, had substantial levels of CAR detectable. This suggested that the CAR receptor was important to vector infection in cell lines.

All tumor cell lines studied had high levels of the alpha v beta 3 and alpha v beta 5 receptors in the cell lines with lower levels of LacZ gene expression were seen when we restrict our analysis to cell lines with substantial level of CAR (Ovcar-5, Skov3, EJ, J82). The observed low level of the alpha v beta 3 integrin receptor in Nutu-19 cell line, which shows high level of adenoviral CMV-LacZ gene expression may again reflect the amino acid sequence divergence of the rat integrin receptor from the human integrin receptor, making this receptor in the rat cell non- reactive with an anti-human integrin receptor antibody. Our conclusion is that the CAR is important for Ad-CMV-LacZ vector infectivity and transgene expression.

Comparison of Lac Z Gene Expression Levels in Cell Lines Infected with the Ad-CMV-LacZ and Ad-Lp-LacZ Vectors: In order to determine if 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 for the percentage of cells in which the presence of beta-galactosidase was detectable in the NuTu-19, Ovcar-5, EJ, J82, Skov3, Hey and CCD cell lines following exposure to the Ad-CMV-LacZ and Ad-Lp-LacZ vectors. We then calculated a ratio of the percentage of cells positive for beta-galactosidase in the CMV to the LP transcription unit bearing vectors, to normalize the data for differences in CAR and integrin receptors in each cell line. As shown in Table 2, the ratio of CMV to LP expression 5 days following exposure to each

vector at 80 MOI was highest (10/1) with the CCD cell line, whereas the ratio in epithelial neoplastic cell lines was much lower (4/1, 1/1, 1.4/1, and 1/1). The relatively low percentage of Lac Z gene expression in the CCD cell line exposed to the Ad-Lp-Lac Z vector is not due to low infectivity by the Ad-Lp-Lac Z vector since as shown in Table 2, the percentage of CCD cells positive for beta-gtalactosidase following exposure to the Ad-CVM-Lac Z vector is 95%. These data suggest that the L-plastin promoter is more active in epithelial neoplastic cell lines than in minimal deviation fibroblast cell lines.

Time Course of Expression of Adenoviral Transgenes after Infection When Driven by the CMV vs the Lp Promoter: 6×10^5 cells were infected with the Ad-CMV-LacZ and Ad-LP-LacZ vectors at different MOI for 90 minutes. The cells were then seeded in 6 well plates in duplicate and then stained by the X-Gal reaction at 2, 3, 4, 5, 6, and 7 days following the infection to measure the percentage of beta-galactosidase positive cells at each time point. The expression of the Lac Z transgene vector quickly reached a maximum following exposure to the Ad-CMV-Lac Z and persisted at that level for 3-5 days (80MOI) before it started to decrease. When a lower MOI (5MOI) was used, the maximum expression level persisted a shorter time. Cells infected with the Ad-Lp-LacZ vector reached a maximum level of expression of the Lac Z gene at a much later time than was the case with Ad-CMV-LacZ infected cells. Although the levels of beta-galactosidase in cells exposed to the Ad-Lp-Lac Z was initially less than that seen in the same cells exposed to the Ad-CMV-Lac Z vector, ulitmately, the levels

generated by the Ad-Lp-Lac Z ultimately reached a maximum at day 3 (80MOI), which was equal to that seen with the Ad-CMV/Lac infected cells (see Figure 2, the EJ cell line).

Studies of the Effect of 5-FU Released from Infected Cells on Non-Infected Cells: In order to monitor the effect of 5FU released from infected cells on the survival of non-infected cells, mixtures of Ad-CMV-CD or Ad-Lp-CD vector infected and non-infected cells were generated and then exposed to 5FC. When as few as 5% of the population of Ovcar-5, or NuTu-19 ovarian cancer cell lines or the CCD minimum deviation fibroblast cell line cells infected with Ad-CMV-CD (20MOI) vectors were mixed with 95% of uninfected cells, the majority of the cells were lost when cells were exposed for 6 days to 5FC at a 500umol concentration (see Figure 3). 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 which are required to kill the vast majority of infected as well as uninfected tumor cells. When the Ovcar-5, NuTu-19 and CCD 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, all the cells were eradicated at the highest MOI when similar experiments were carried out with the Ad-Lp-CD vector with the Ovcar-5 and Skvo3 ovarian carcinoma cell lines. This indicates that the Lp promoter is more active in the epithelial neoplastic cell lines than in the CCD minimum deviation cell line.

5FU Sensitivity of Each Cell Line Expressed as IC50: The intrinsic sensitivity of each cell line to 5FU was then tested by seeding 3×10^5 cells in T25 flasks in triplicate, and incubated for 96 hours at different 5FU concentrations. The IC50 to 5FU for J82 is 55uM, EJ is 30uM, Nutu-19 is 5uM, Ovcar-5 is 3uM, Skov3 is 22uM , Hey is 85uM and CCD is 15uM. The IC50 of the CC D cell line (15 uM) is less than that of several of the epithelial neoplastic cell lines (EJ, J82, Svok3). Thus, the low sensitivity of the CCD fibroblast cell line to the effect of the Ad-Lp-CD vector/5FC treatment is therefore not due to a high level of resistance to 5FU.

Study of the Toxicity of the Adenoviral Vector Backbone without the CD Gene: In order to test how much of the toxicity of the Ad-Lp-CD/5FU treatment seen in Figure 3 was due to the vector backbone and how much was due to 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. Following this, the cell lines were incubated in medium supplemented with 500uM 5-FC for 5 days. As shown in Figure 4, much less toxicity was seen at all MOI with the Ad-CMV-LacZ or Ad-Lp-LacZ vectors than was the case with the Ad-CMV-CD or Ad-Lp-CD vectors. Thus, the majority of the toxicity seen in these experiments was not due to the viral backbone, but due to the effect of the CD transcription units on the conversion of 5FC to 5FU.

LacZ Expression in Monolayer Cultures of Samples Obtained at Surgery from Normal Peritoneum and Metastatic Implants of Ovarian Cancer Following Exposure to Ad-Lp-

LacZ and 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 dissegregate the tissue. The resulting cells were then cultured in RPMI-1640 supplemented with 10% NBCS. Following culture, the cells were exposed at a MOI of 20 to the Ad-CMV-LacZ or Ad-Lp-LacZ vectors in the T-flasks for 90 minutes. After 48 hours of incubation, the infected cells were measured by X-gal staining or FACS. All experiments were performed at 80% confluency. As shown in Figure 5, in situ staining of these cultures showed a blue color in all but the culture of normal peritoneum which had been exposed to the Ad-Lp-LacZ vector.

The percentage of X-gal positive cells in different patient samples following exposure to the Ad-CMV-LacZ vector at 80 MOI, as determined by FACS, was calculated to be: ascites:50-80%, primary tumor:50-90%, metastatic tumor:45-85%, and normal peritoneum:60%. When cells were exposed to the Ad-Lp-LacZ at 80 MOI, the X-Gal staining in ascites was 10-35%, primary tumor 15-60%, metastatic tumor 15-45%, and normal peritoneum 1%. As shown in Table 3, the ratio of X-gal staining with CMV/LP vectors was highest with the normal peritoneum (60/1), whereas this ratio was in the range of 1/1 to 3/1 in the case of ovarian cancer. The use of the ratio of CMV/L-plastin normalizes the levels of expression of vector transgenes for variables related to infection of these cells by the vectors. These results indicate that the normal peritoneum is less able to support the expression of transgenes driven by the LP promoter than in ovarian cancer cells.

Cytotoxicity of Ad-CMV-CD and Ad-Lp-CD Vectors in Monolayer Cell Cultures of Normal Peritoneum and Ovarian Cancer from Surgical Specimems: Samples of primary tumor, metastatic tumor and normal peritoneum were collected from 16 ovarian cancer patients, and samples were prepared with 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 500uM 5FC in T25 flasks, 98% of the ovarian cancer ascitic cells were killed by the Ad-CMV-CD vector and 5FC, and 85% of the cells were killed by the Ad-Lp-CD vector/5FC system. In the primary tumor cells, 90% of the cells were killed by the Ad-CMV-CD vector and 75% were killed with Ad-Lp-CD vector. In metastatic cells, 85% were killed by the Ad-CMV-CD vector and 70% were killed by the Ad-Lp-CD vector. In contrast to the results with 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 $1/60^{th}$ of that seen with the Ad-CMV-CD vector. This indicates that the expression of the Lp-CD gene is much lower in the CCD cell line than in the ovarian cancer cell lines.

Studies of CD and LacZ Vectors in Organ Cultures of Normal Ovary: The samples of ovarian cancer and normal ovary tissues were cut into small pieces and were then inoculated in organ culture for 24-48 hours, infected with either the Ad-CMV-LacZ or the Ad-LP-LacZ vectors for 48 hours, and then the cells were processed with the X-gal staining reaction. The organ culture differs from the monolayer culture in that the

organ culture is a 3 dimensional array of cells. As shown in Figure 6, 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 than with the Ad-Lp-LacZ vector. Again, the results indicate that the CMV promoter is much more active in normal tissue than is the Lp promoter.

Killing Efficiency of Ovarian Cancer Tumor Cell Lines by 5FC/CD Vector System in Nude Mice: In order to test the in vivo efficacy of the Ad-Lp-CD replication incompetent vector system, we infected ex vivo either the Skov3 ovarian cancer cell line at 80 MOI or the Ovcar-5 ovarian cancer cell line ex vivo at 100 M0I by incubating the cells in the vector for 60 minutes. Then we injected 40 million of these infected cells into 10 SCID mice with the Ovcar-5 ovarian cancer cell line or 5 SCID mice with the Skov3 ovarian carcinoma cell line. One day after the injection of the tumor cells, we initiated daily intraperitoneal injections of each of the animals with 5FC to generate daily peak intraperitoneal 5FC concentrations in the 500 uM range. We carried on the daily intraperitoneal 5FC injections for 10 days after the tumor injection. At 21 days after injection into the mice, we sacrificed 7 of the Ovcar-5 injected mice and all 5 of the Skov3 injected mice and examined the peritoneal cavity for tumors. The remaining 3 Ovcar-5 mice that were not sacrificed at 21 days were sacrificed at 50 days after tumor injection. 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 10 of the animals injected with the Ovcar-5 cell lines and all 5 of the animals injected with the Skov 3 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 SCID mice if all of the tumor cells are infected 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. The peritoneal surface was totally normal in these animals both at the gross and microscopic level, showing no sign of mononuclear cell inflammation. This data shows that the toxic effect of the vector/5FC system is specific for the tumor cells, and that there is no bystander effect damage on the normal cells of the peritoneum.

Discussion: Our cell line studies indictated that adenoviral vectors carrying transcription units under the control of the L-plastin promoter initially generate lower levels of transgene expression in cells exposed to these vectors than seen when adenoviral vectors which carry transcription units governed by the CMV promoter. However, from the data presented on the time course of expression of the L-plastin promoter controlled LacZ transcription units, the final level of expression of the L-plastin controlled transcription unit was equal to that achieved by the CMV transcription units in adenoviral vectors.

In addition, when the L-plastin promoter is used to drive the expression of CD chemotherapy sensitization transcription unit, the amount of intracellular CD protein which is generated by the Ad-Lp-CD vector in ovarian and bladder cancer cell lines is

sufficient to result in killing of 100% of all of the epithelial neoplastic cell lines, when only 5-50% of the cells are infected. In contrast, the toxicity generated in the CCD fibroblast cell lines is much lower, never reaching 100%, presumably due to lower levels of activity of the L-plastin promoter in the CCD cell line (see Figure 3). Control experiments have shown that the CCD cell line is infectable by the Ad-CMV-LacZ, so that low infectivity is not responsible for the low sensitivity to the Ad-Lp-CD vector. In addition, the intrinsic sensitivity of the CCD to 5-FU directly added to the culture is not lower than the majority of the neoplastic cell lines. Thus, it appears that the level of expression of the Lp driven genes in the CCD is lower than that seen in the ovarian 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 neoplastic cells show that the ratio of cytotoxicity of CMV-CD adenoviral vectors to L-plastin CD adenoviral vectors is highest in normal peritoneum (60) as compared to 2-6 in malignant ascites, or primary and metastatic ovarian cancer. Finally, the use of the Ad-Lp-CD vector to infect ovarian cancer cell lines prior to their injection into intraperitoneal cavity results in a suppression of engraftment of these ovarian cancer cell lines in SCID mice, whereas no sign of suppression of tumor growth occurred when the ovarian cancer cell lines were infected with Ad-Lp-LacZ or Ad-CMV-LacZ vectors. No sign of inflammation of the peritoneal surface was seen in these animals.

These data suggest that, in principle , the L-plastin CD transcription units may be 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 which 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 Figure 6 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 pre-clinical level of several different types of adenoviral vectors which 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. Such vectors may have utility in the intraperitoneal therapy of ovarian cancer.

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Figure Legends:

Fig.1. Light Microscopic Study of the LacZ Gene Expression levels in Different Cell Lines. $2x10^5$ cells were exposed to the Ad-LP-LacZ or Ad-CMV-LacZ viral vectors for 90 minutes. After 48 hours, X-Gal staining was performed. The positive cells were counted in the high powered field of a light microscope. A: CCD cell line, Ad-CMV-LacZ at 20 MOI, 200X; B: EJ cell line, Ad-CMV-LacZ at 20MOI, 200X; C: CCD cell line, Ad-LP-LacZ at 80MOI, 100X; D: EJ cell line Ad-LP-LacZ at 80MOI , 100X.

Fig.2. Time Course of Beta-Galactosidase Expression after Exposure to the Ad-CMV-LacZ and Ad-LP-LacZ Vectors in Different Cell Lines. $6x10^5$ cells were infected at different MOI with the Ad-CMV-LacZ and Ad-LP-LacZ vectors. Then cells were seeded in 6 well plates in duplicate. X-Gal staining was performed at day 2,4,5,6,7 and the positive cells were counted in the high power field of a light microscope.

Fig. 3. Toxicity of Vectors at Varying Levels of Infected Cells. Different cell lines were infected at varying MOI (20, 80, 160) using the Ad-CMV-CD or Ad-LP-CD viral vectors. The infected cells and non-infected cells were mixed in varying ratios to generate 0, 5%, 20%, 30%, 40%, 50%, 60%, 100% infected cells. Cells were then seeded in 6 well plates and incubated for 5 days in 500umol 5-FC. The cells were then trypsinized and surviving cells were counted by Trypan Blue exclusion.

Fig.4. Study of the Toxicity of the Control Lac Z Vector vs the CD Vector. $2x10^5$ cells were infected at 0, 5, 20, 40,80 and 160 MOI vector with the Ad-CMV-LacZ, Ad-Lp-LacZ, Ad-CMV-CD or Ad-Lp-CD vectors for 90 minutes and seeded in 6 well plates in duplicate. Cells were then incubated in 500umol 5FC for 5 days. The percentage of surviving cells was counted by Trypan Blue exclusion.

Fig.5. Primary Cell Cultures. Samples of tumor tissues and normal peritoneum were collected from 16 ovarian cancer patients undergoing diagnostic or therapeutic laparotomy and cut into small pieces. These pieces were then digested with collagenase to produce tissue disaggregation and the resulting cells cultured in RPMI1640 with 10% fetal calf serum. When the cells were at 80% confluency, they were exposed in the flasks for 90 minutes to either the Ad-Lp-LacZ or Ad-CMV-LacZ vectors. After 48 hours of incubation, the Lac Z beta-galactosidase positive cells were measured by X-Gal staining.

Fig.6. 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 with 10% charcoal-stripped serum. 24-48 hours later, the tissues were infected with vectors for 90 minutes, PBS washed and then incubated 48 hours. Then the tissues were frozen in O.C.T following which the frozen section were stained by the X-Gal reaction. Left Panel: control; Middle Panel: Ad-CMV-LacZ vector; Left Panel: Ad-Lp-LacZ vector.

Table 1. The Relationship Between the CAR, or $\alpha v\beta 3$, $\alpha v\beta 5$ Receptor Expression Level and Infectivity by the Ad-CMV- LacZ Vector as Measured by LacZ Expression(Percent Cells Positive for Beta-Galactosidase)

	$\alpha v\beta 3$	$\alpha v\beta 5$	CAR	Ad-CMV-LacZ
Ovarian Cancer Cell Lines:				
Nutu-19	17.37*	93.79	10.2*	100
Skov3	63.75	91.2	87.25	85
Ovcar-5	47.71	57.15	88.07	65
Hey	81.3	96.3	0.0	10
Bladder Cancer Cell Lines:				
EJ	82.73	81.53	94.7	95
J82	55.8	77.9	80.3	88
Minimal Deviation Cell Line:				
CCD	62.8	92.8	29	70

$\alpha v\beta 3$,$\alpha v\beta 5$ and CAR (Coxsackie B virus-adenovirus receptor) monocolonal antibodies and the FITC conjugated Anti-Mouse antibody were used to stain the cells. Then, FACS analysis was used to detect the percentage of the positive cells. *Low values probably due to nonreactivity of the mouse antihuman CAR or integrin monoclonal antibody used with the rat receptors.

Table2. Comparison of Positivity Percentage for LacZ Gene Expression in Ovarian Cancer Cells Exposed to Either the Ad-CMV-LacZ versus the Ad-Lp-LacZ Vectors.
(Percentage of LacZ Gene Expression)

	Ad-CMV-LacZ				Ad-Lp-LacZ				
	Day3		Day5		Day3		Day5		
	20MOI	80MOI	20MOI	80MOI	20MOI	80MOI	20MOI	80MOI	CMV/LP
Ovarian Cancer Cell Lines:									
Skov3	85	95	15	40	25	60	8	35	1/1
Ovcar-5	65	80	10	35	10	35	1	8	4/1
Hey*	10	35	5	10	0	2	0	0	----
Bladder Cancer Cell Lines:									
EJ	95	100	45	85	55	95	45	60	1.3/1
J82	90	100	15	35	20	55	7	35	1.1/3
Minimal Deviation Fibroblast Cell Lines:									
CCD	70	95	12	55	3	8	2	5	10/1

Light microscopic study of the LacZ gene expression levels in different cell lines exposed to either the Ad-CMV-LacZ or Ad-Lp-LacZ vectors. 2×10^5 cells were infected at 20 and 80 MOI with the Ad-LP-LacZ or Ad-CMV-LacZ viral vector for 90 minutes. Then the cells were incubated in 6 well plates in duplicate. 48 and 96 hours after exposure, the X-Gal staining was performed. The positive cells were counted through the use of the light microscope. The results are presented as the percentage of cells positive for beta-galactosidase. *Cell line is negative for the CAR receptor needed for infection of cells by the adenoviral vector.

Table3. LacZ Transgene Expression Efficiency in Primary Cultures of Ovarian Cancers and Normal Peritoneal Cells

Vector	Assay	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		3/1	1/1	3/1	60/1

Samples of primary tumor, metastatic tumor and normal peritoneum were collected from 16 ovarian cancer patient undergoing diagnostic or therapeutic laparotomy and cut into small pieces. These pieces were then digested with collagenase to produce tissue disaggregation and the resulting cells cultured in RPMI with 10% NBCS. All experiments were performed at 80% confluency. 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 minutes, after 48hours incubation the positive cells were mesured by X-gal staining or FACS.

Table 4. Cytotoxicity in Monolayer Culture of Normal Peritoneum and Ovarian Cancer Cells Following Expression to Ad-Lp-CD and Ad-CMV-CD Vectors and 5FC

	Ad-CMV-CD	Ad-Lp-CD
Ascites	98%	85%
Metastatic Tumor	85%	70%

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

Table 5. Tumor Growth In Animals Injected with Adenoviral LP Vectors
(Percent of Animals Found to be Positive for Tumors)

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

The SCID mice were injected with 40 million Ovcar-5 or Skov3 tumor cells which were had been previously infected in vitro with the Ad-Lp-LacZ vector or the Ad-Lp-CD vector. Starting on the second day, 500mg/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.

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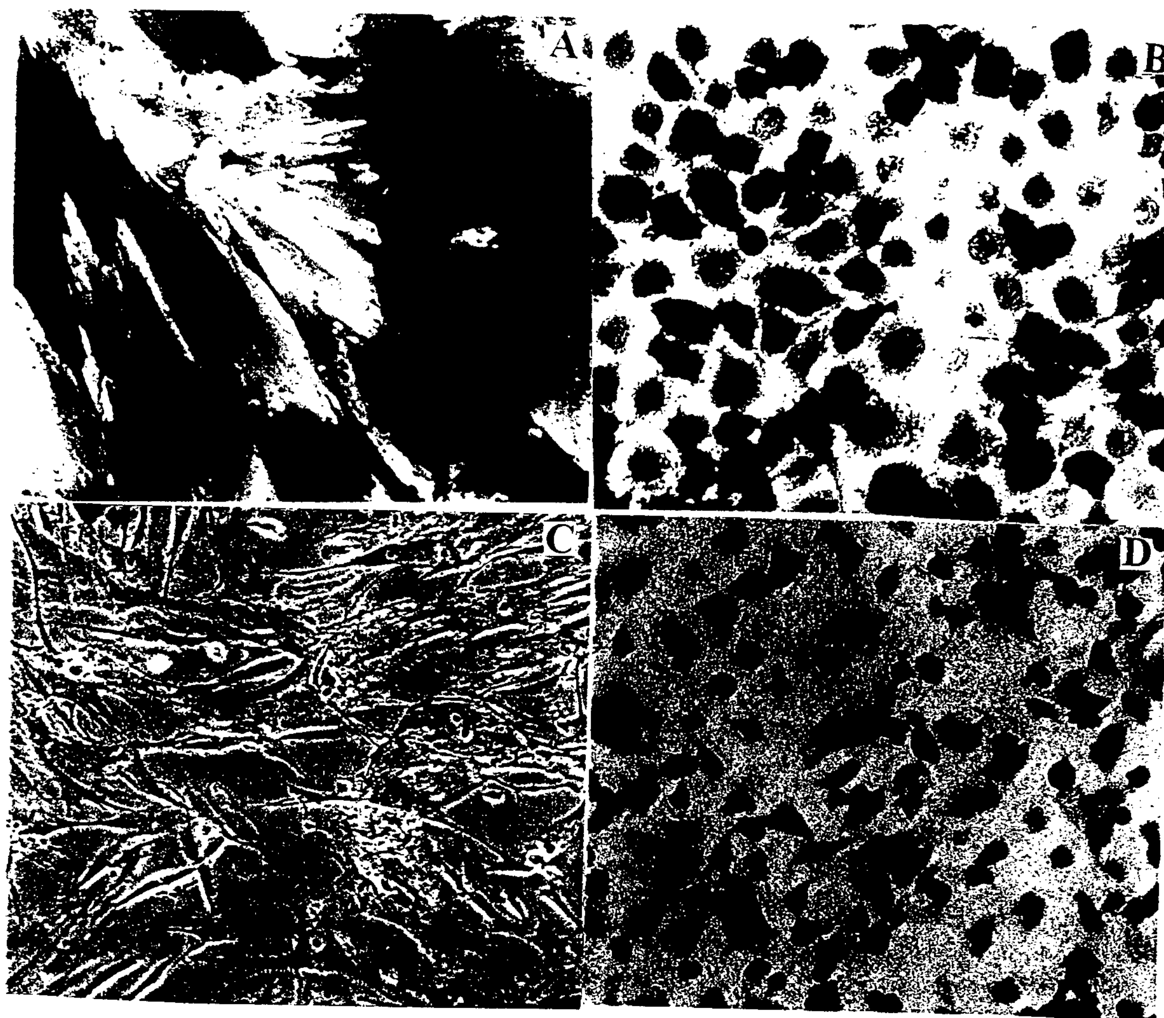

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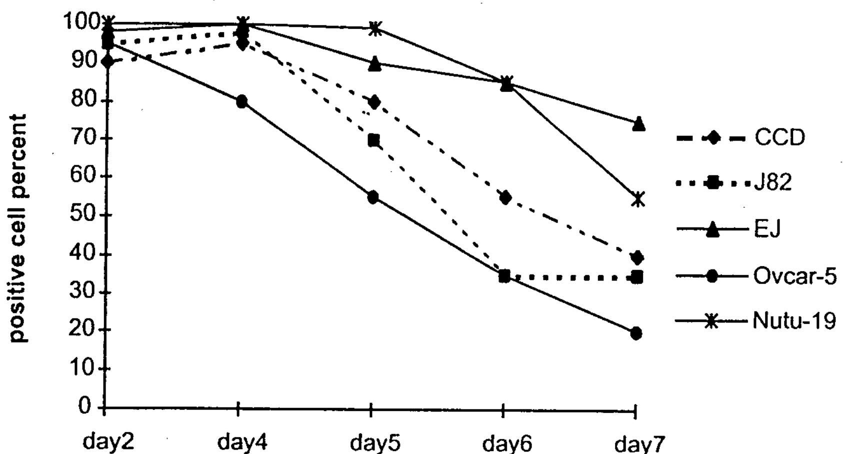

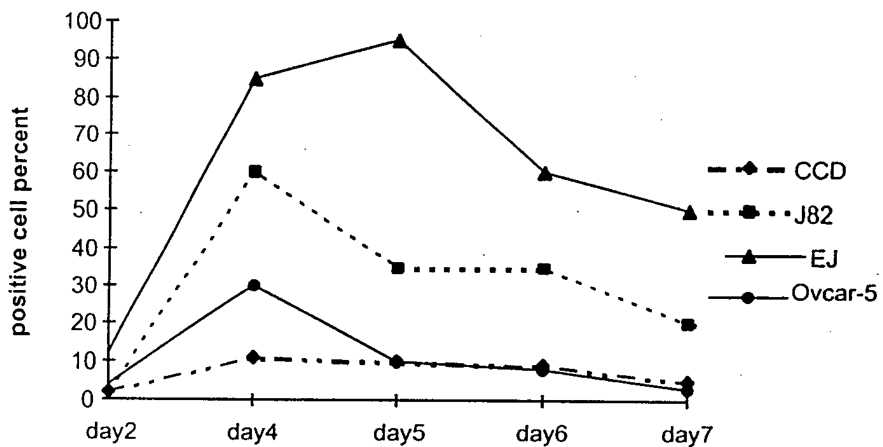

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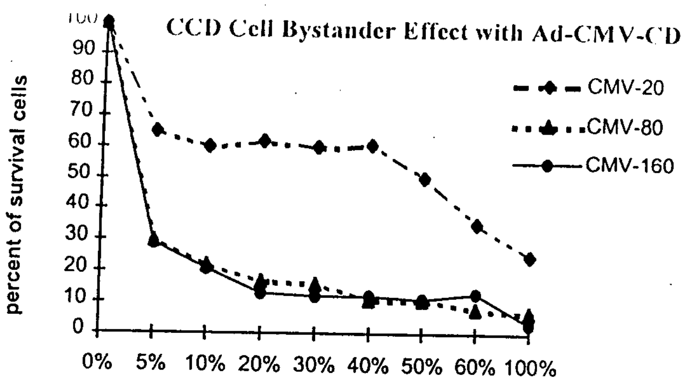

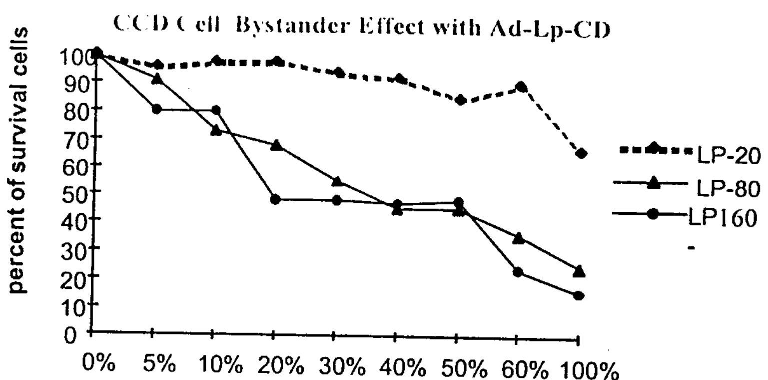

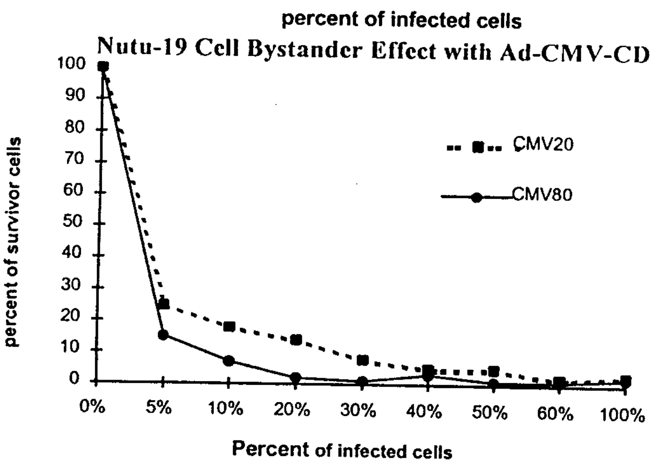

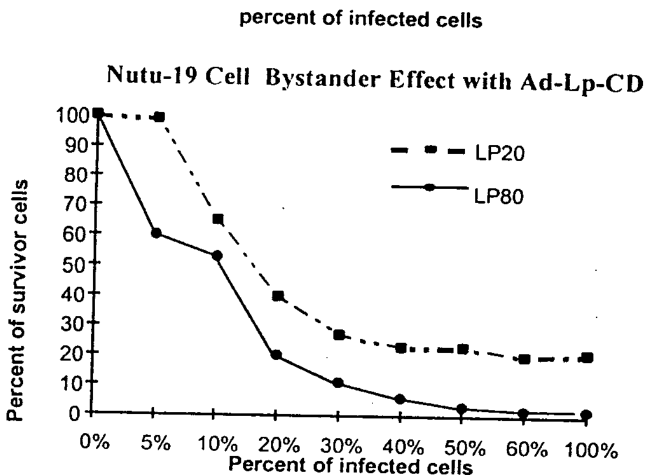

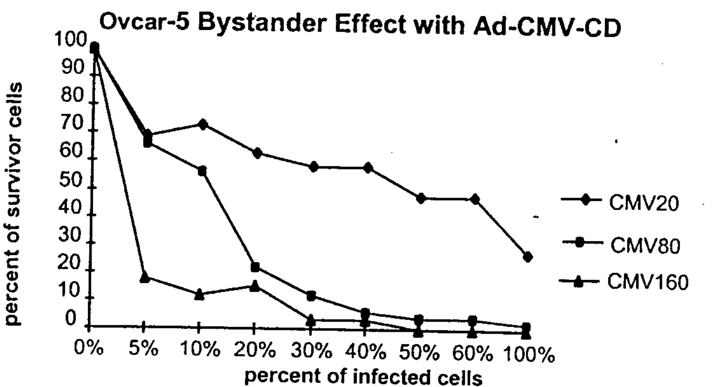

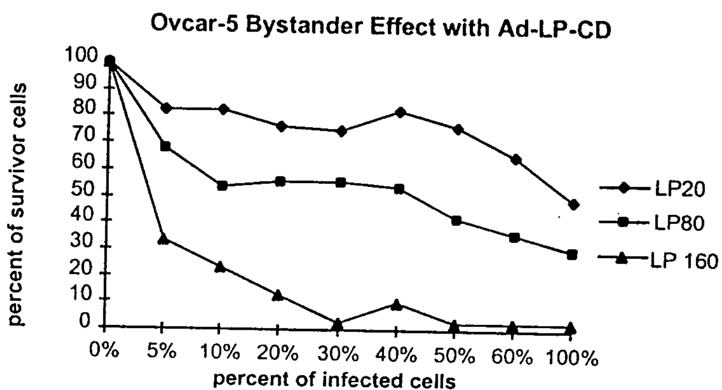

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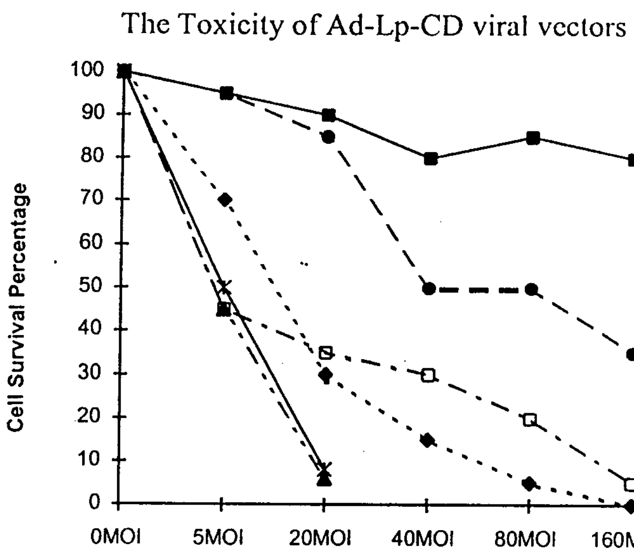

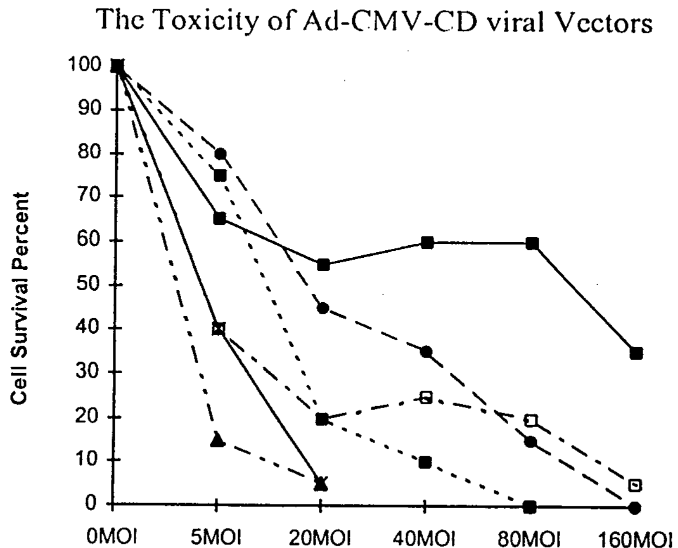

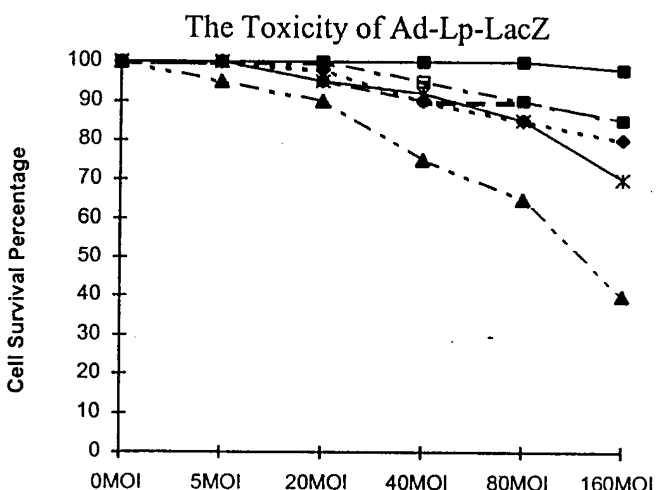

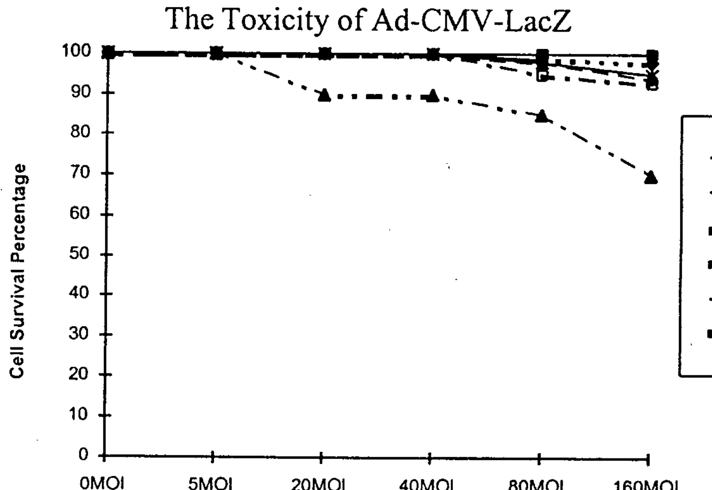

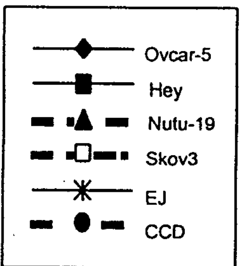

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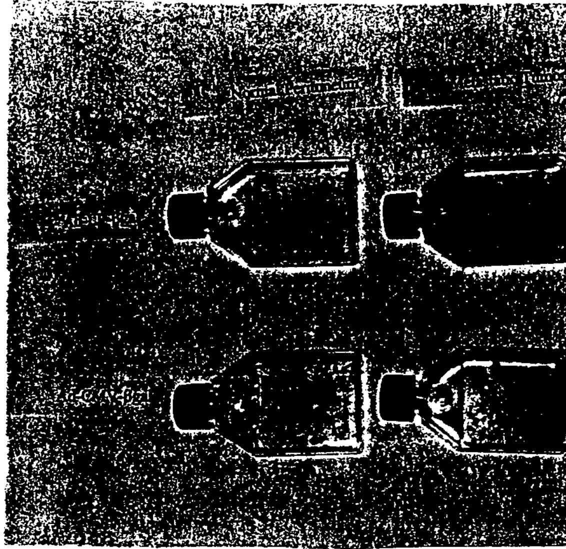

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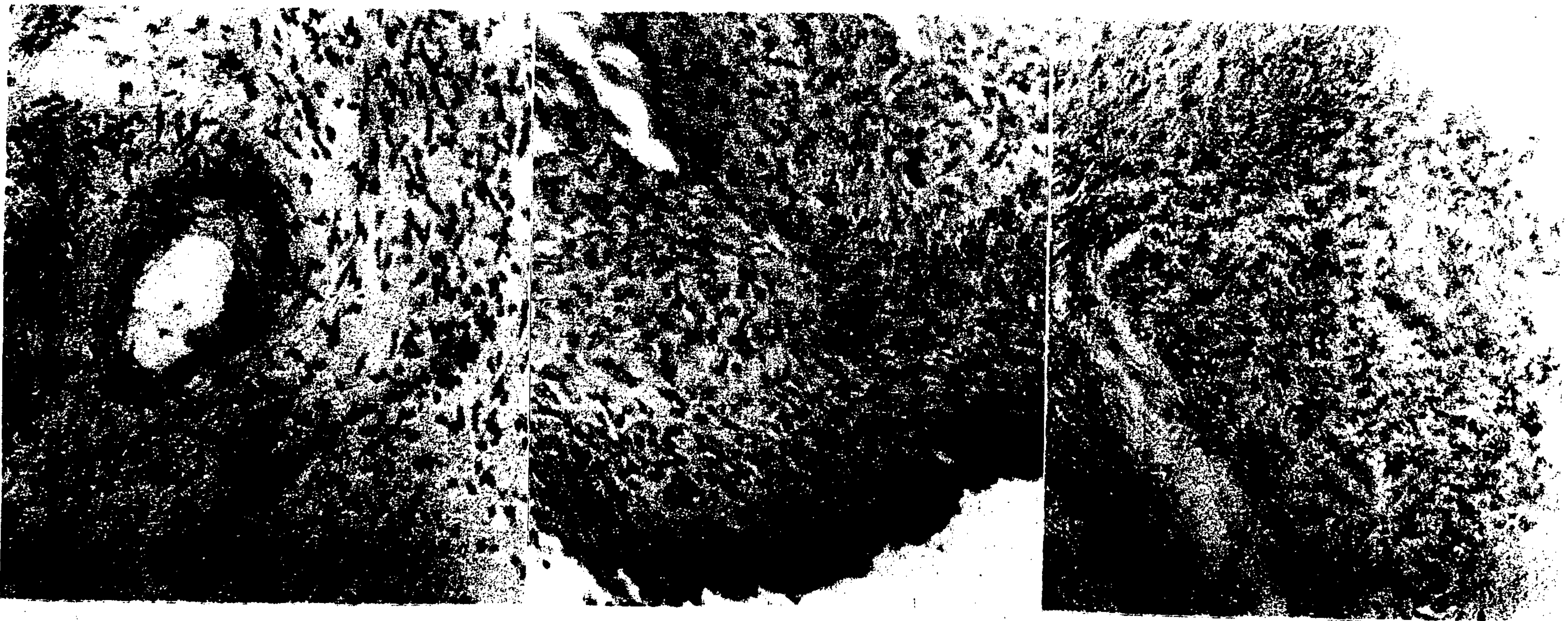

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