

## Targeted Tumor Killing via an Intracellular Antibody against erbB-2

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## Abstract

Specific killing of erbB-2-overexpressing tumor cells can be achieved using expression of an intracellular antibody directed against the erbB-2 oncoprotein. We have developed a strategy using a recombinant adenovirus encoding an anti-erbB-2 single chain antibody to achieve targeted tumor cell killing in vivo and can show significantly prolonged survival of animals carrying a human ovarian carcinoma tumor burden within their peritoneal cavities. This strategy of gene therapy for ovarian carcinoma offers the potential to achieve highly specific, targeted killing of human tumor cells and thus establishes the rationale to undertake human clinical trials on this basis. (*J. Clin. Invest.* 1995. 96:2980–2989.) Key words: ovarian carcinoma • erbB-2 • sFv • gene therapy • antibody

## Introduction

Strategies have been developed to accomplish gene therapy for cancer based upon specific correction of the genetic lesions associated with neoplastic transformation. In this regard, methods have been proposed to achieve the augmentation of tumor suppressor genes which have undergone mutational inactivation by replacement of their wild-type antioncogene function (1–4). In addition, specific approaches have been developed to accomplish targeted abrogation of overexpressed dominant oncoproteins. These approaches have been based largely upon the employment of antisense methods to achieve selected knockout of a target oncoprotein at the transcriptional (5, 6) or posttranscriptional level (7–9).

As an alternate means to achieve abrogation of transforming oncogenes, we have developed a method based upon expression

of an intracellular single-chain antibody (sFv) directed against the target oncoprotein (10). In the context of the erbB-2 tyrosine kinase receptor, we have demonstrated that sFv-mediated entrapment at the level of the endoplasmic reticulum (ER)<sup>1</sup> can trigger cellular apoptosis (11). Importantly, this effect is accomplished selectively in erbB-2-overexpressing tumor targets; the viability of non-erbB-2-expressing cells is not affected by this genetic intervention (11, 12). This approach thus represents a novel genetic means to accomplish a highly selective, targeted tumor cell killing.

In this report, we have further explored the utility of this gene therapy strategy in ovarian carcinoma. We show here that delivery of an anti-erbB-2 sFv gene offers an approach which allows selective tumor cell killing, with a therapeutic outcome, in a murine model of human ovarian carcinoma. These promising findings provide the rationale to undertake human clinical trials.

## Methods

**Cell lines.** The human ovarian carcinoma cell line SKOV3. ip1, an SKOV3 derivative cell line, was kindly provided by Janet Price (Baylor University, Houston, TX). This cell line was maintained in DME/F12 media supplemented with 10% heat-inactivated FCS (PAA, Linz, Austria), L-glutamine (200 µg/ml), penicillin (100 U/ml), and streptomycin (25 µg/ml) at 37°C, in a humidified 5% CO<sub>2</sub> atmosphere.

**Determination of an optimum vector for in vivo transduction.** 10<sup>7</sup> SKOV3. ip1 cells were transplanted orthotopically into athymic nude mice by the intraperitoneal route. 48 h after transplantation, animals were challenged with vectors encoding an *Escherichia coli* β-galactosidase reporter gene (LacZ). Animals (seven per group) received 100 pfu/cell of AdCMVLacZ, AdpL complex containing a LacZ-encoding plasmid pCMVβ, or DOTAP–DNA complexes containing pCMVβ. Preparation of AdpL complexes and DOTAP–DNA complexes has been described elsewhere (11, 13, 14). Peritoneal lavage was performed 48 h later to harvest the tumor cells. Red blood cells were lysed with the ACK lysis buffer (0.15 M NH<sub>4</sub>Cl, 1.0 mM KHCO<sub>3</sub>, 0.1 mM Na<sub>2</sub>EDTA, pH 7.4). Lysis was terminated by the addition of 5 ml of complete media. The tumor cells were then analyzed by FACS® for reporter gene-transduced cells.

Briefly, 10<sup>6</sup> tumor cells were incubated with biotinylated polyclonal anti-erbB-2 antibody (Dako Corp., Carpinteria, CA) for 30 min at room temperature. Cells were then washed (3 × 20 min) with PBS (pH

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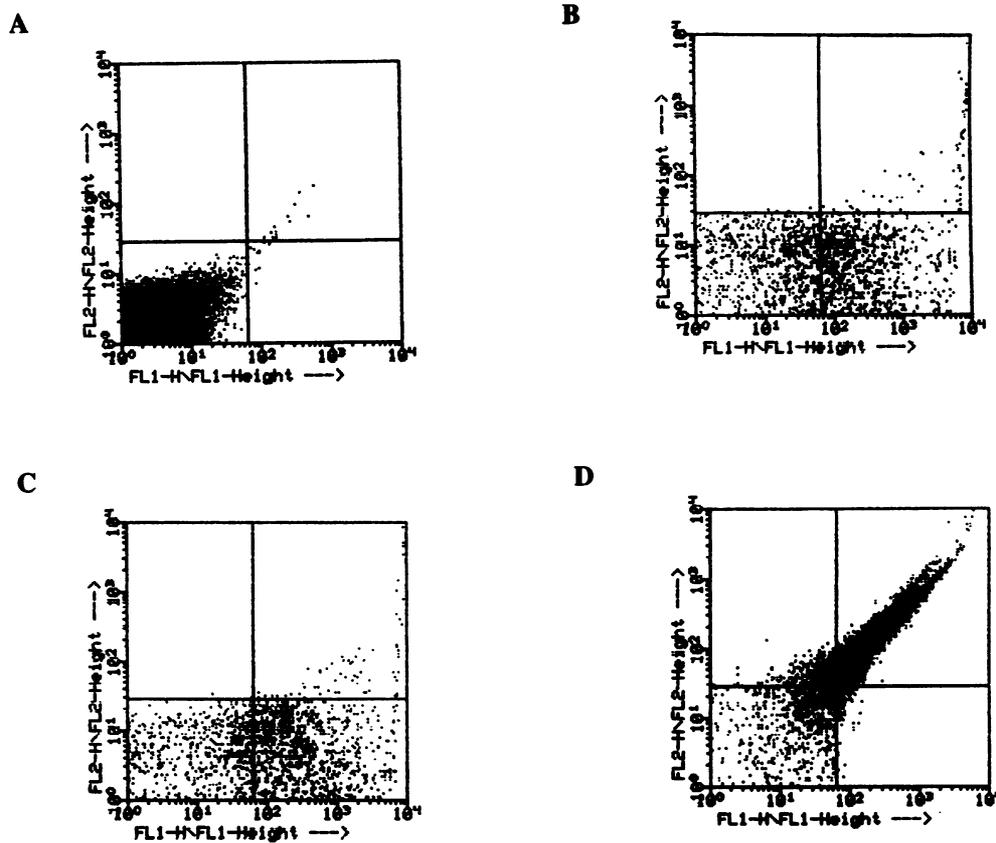
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1. **Abbreviations used in this paper:** ER, endoplasmic reticulum; HSVtk, herpes simplex virus thymidine kinase.



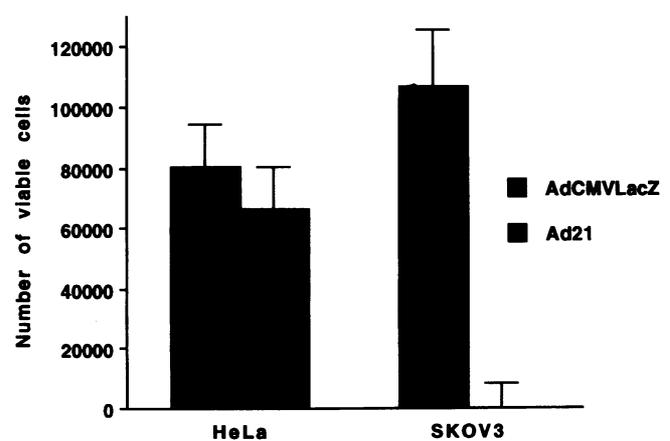
**Figure 1.** Determination of optimal vector strategy to accomplish in situ transduction of tumor cells localized in the peritoneum. Various vectors encoding the *E. coli*  $\beta$ -galactosidase reporter gene (LacZ) were delivered by the intraperitoneal route to athymic nude mice xenografted 48 h previously with the human ovarian carcinoma cell line SKOV3. ip1. Evaluated vectors included a E1A/B-deleted replication incompetent adenovirus, Ad-CMV $\beta$ LacZ; adenovirus-polylysine-DNA complexes containing the LacZ-encoding plasmid pCMV $\beta$  and DOTAP-DNA complexes, also containing the plasmid pCMV $\beta$ . After transduction (48 h) animals underwent lavage to harvest peritoneal cells, which were then sorted by FACS<sup>®</sup> for cell surface erbB-2 and LacZ expression. Experimental groups included: A, no vector transduction; B, DOTAP-DNA complexes; C, adenovirus-polylysine-DNA complexes; D, recombinant adenovirus.

7.4) and incubated with streptavidin-phycoerythrin (kindly provided by Dr. Pat Bucy, University of Alabama at Birmingham). Cells were washed again with PBS and resuspended in staining media (10 mM HEPES and 4% FCS in PBS) in a 6-ml FACS<sup>®</sup> tube (Falcon, Franklin Lakes, NJ) and warmed at 37°C for 10 min. Prewarmed 2 mM fluorescein-di-galactoside (FDG) (100  $\mu$ l) was added, and the cells were incubated at 37°C for exactly 1 min. FDG loading was stopped by the addition of 1 ml of cold staining media. Cells were kept on ice and in the dark until analysis was completed. 10,000 cells were size-sorted first and then sorted for erbB-2-positive and LacZ-positive cells.

**In vitro efficacy of the recombinant adenovirus encoding anti-erbB-2 sFv.** A replication-incompetent, E1-deleted recombinant adenovirus encoding the ER form of the anti-erbB-2 sFv gene was constructed using homologous recombination techniques (15). This methodology has been described elsewhere (16, 17). SKOV3. ip1, a human ovarian carcinoma cell line overexpressing erbB-2, and HeLa, an erbB-2-non-expressing human cervical carcinoma cell line, were plated at a density of 5,000 cells/well in a 96-well plate. Viral infections were carried out in DME media containing 2% FCS and were allowed to proceed at 37°C for 1 h, after which time complete media were added to each well. Infection was carried at an moi of  $2.5 \times 10^6$  pfu (500 pfu/cell). 96 h after adenoviral infection, cells were analyzed for cell viability by the XTT assay (11). A control adenoviral vector, AdCMV $\beta$ LacZ, was used as a negative control. The XTT assay is based on the ability of viable cells alone to reduce XTT (2,3-bis[2-methoxy 4-nitro-5 sulfophenyl]2-H tetrazolium-carboxanilide salt) to a colored product which can be read spectrophotometrically. At 96 h after infection, the supernatant was removed, and 50  $\mu$ l of solution containing 1.0 mg/ml of XTT, 0.0075 mg/ml phenazine methanesulfate, and 50  $\mu$ l RPMI medium was added to each well. After 30 min, the reduction product was measured at  $A_{450}$  and compared with a standardized curve to determine the number of

viable cells. Tumor cells were also analyzed for cell surface levels of erbB-2 using a polyclonal anti-erbB-2 antibody in combination with ABC-immunoperoxidase detection system (Dako Corp.).

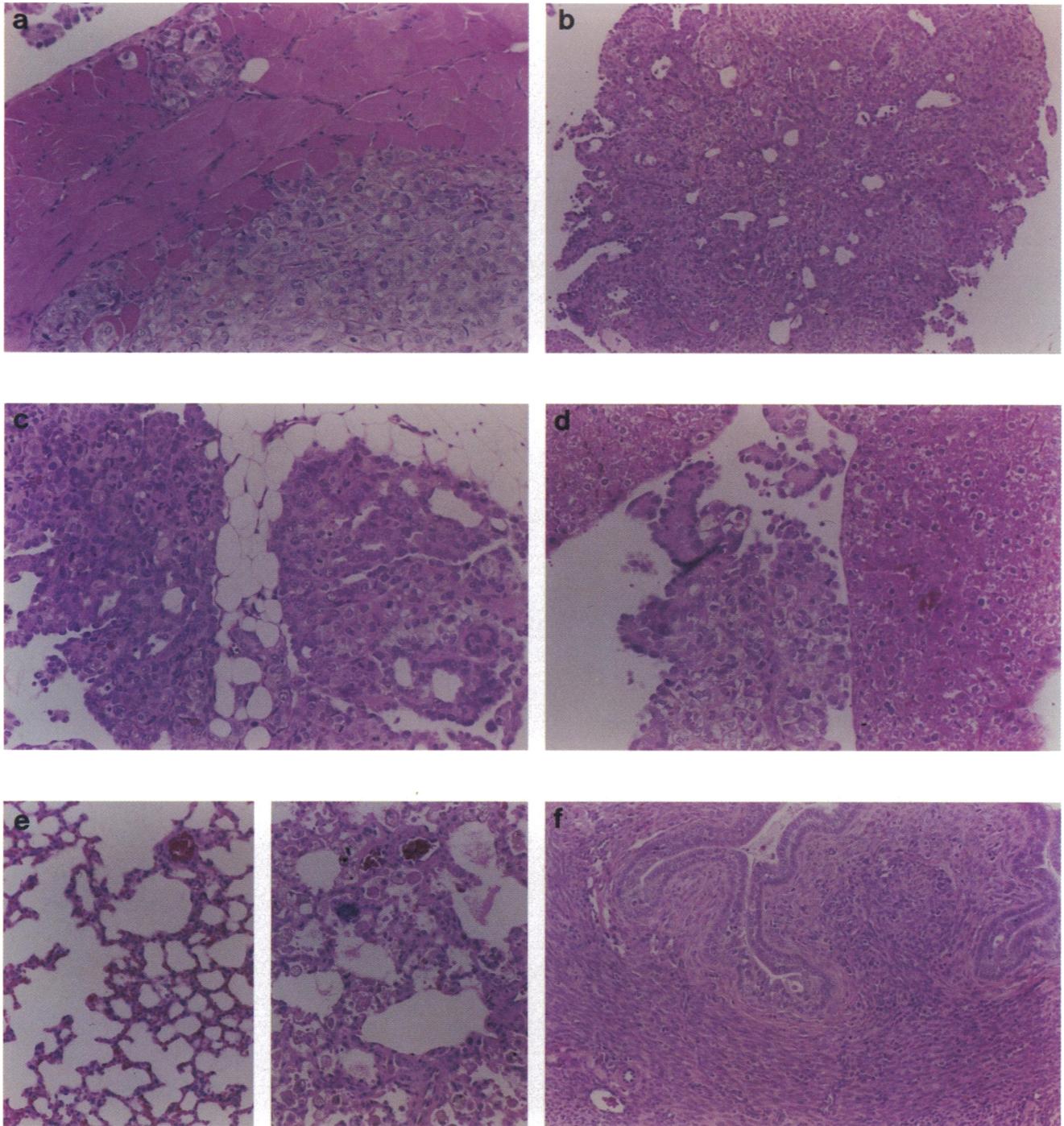
**In vivo efficacy of the recombinant adenovirus encoding anti-erbB-2 sFv.**  $2 \times 10^7$  SKOV3. ip1 cells were transplanted intraperitoneally into athymic nude mice, and tumor cells were harvested by peritoneal lavage after intraperitoneal administration of Ad21 (500 pfu/cell)



**Figure 2.** Analysis of cell viability. Transduced cells were evaluated for viability using the XTT assay at 96 h after transduction. Target cells included the erbB-2-negative cervical carcinoma cell line, HeLa, and the erbB-2-positive ovarian carcinoma cell line, SKOV3. ip1.



# A



**Figure 4.** Antitumor efficacy of anti-erbB-2 sFv encoding adenovirus. CB-17 SCID mice were orthotopically xenografted with  $2 \times 10^7$  SKOV3. ip1 cells and challenged after 48 h with either the reporter gene encoding adenovirus AdCMVLacZ or the anti-erbB-2 sFv encoding adenovirus Ad21. An additional control group received no vector administration. **A** shows representative photomicrographs of control mice receiving no vector. (a) Tumor seen invading into and through abdominal wall musculature. (b) Ovarian carcinoma growing within the peritoneal cavity. (c) Tumor invading perirenal fat. Note attempt at gland formation by malignant cells. (d) Tumor abutting, but not invading the liver parenchyma. (e) *Left*, normal lung parenchyma with minimal congestion; *right*, lung field from another animal with evident pigmented macrophages, interstitial expansion, and focal desquamation. (f) Cross section through the uterine wall. **B** shows representative photomicrographs of mice treated with  $2 \times 10^9$  viral particles of AdCMVLacZ. (a) Ovarian tumor seen attaching to thin strips of peritoneal lining (*top*). (b) Ovarian carcinoma growing within blood vessels. Note loss of normal vascular wall and rare RBCs in the lumen. (c) Ovarian tumor seen invading into the liver parenchyma. (d) Low-power photomicrograph demonstrating normal fallopian tube, uterus, and ovary. (e) Pancreas with prominent central islet of Langerhans. No tumor is present. (f) Normal renal cortex. **C** shows histologic photomicrographs of representative organs and tissue from mice treated with  $2 \times 10^9$  viral particles of Ad21. (a) Ovarian tumor in peripancreatic space. (b) No tumor is seen in the perirenal fat. Note the presence of a small amount of fat necrosis. (c) Lung parenchyma with mild congestion. (d) Calcific pericarditis. (e) Unremarkable murine ovary and fallopian tube. Note prominent ovarian follicles. (f) Mammary gland with normal histomorphology.

## B

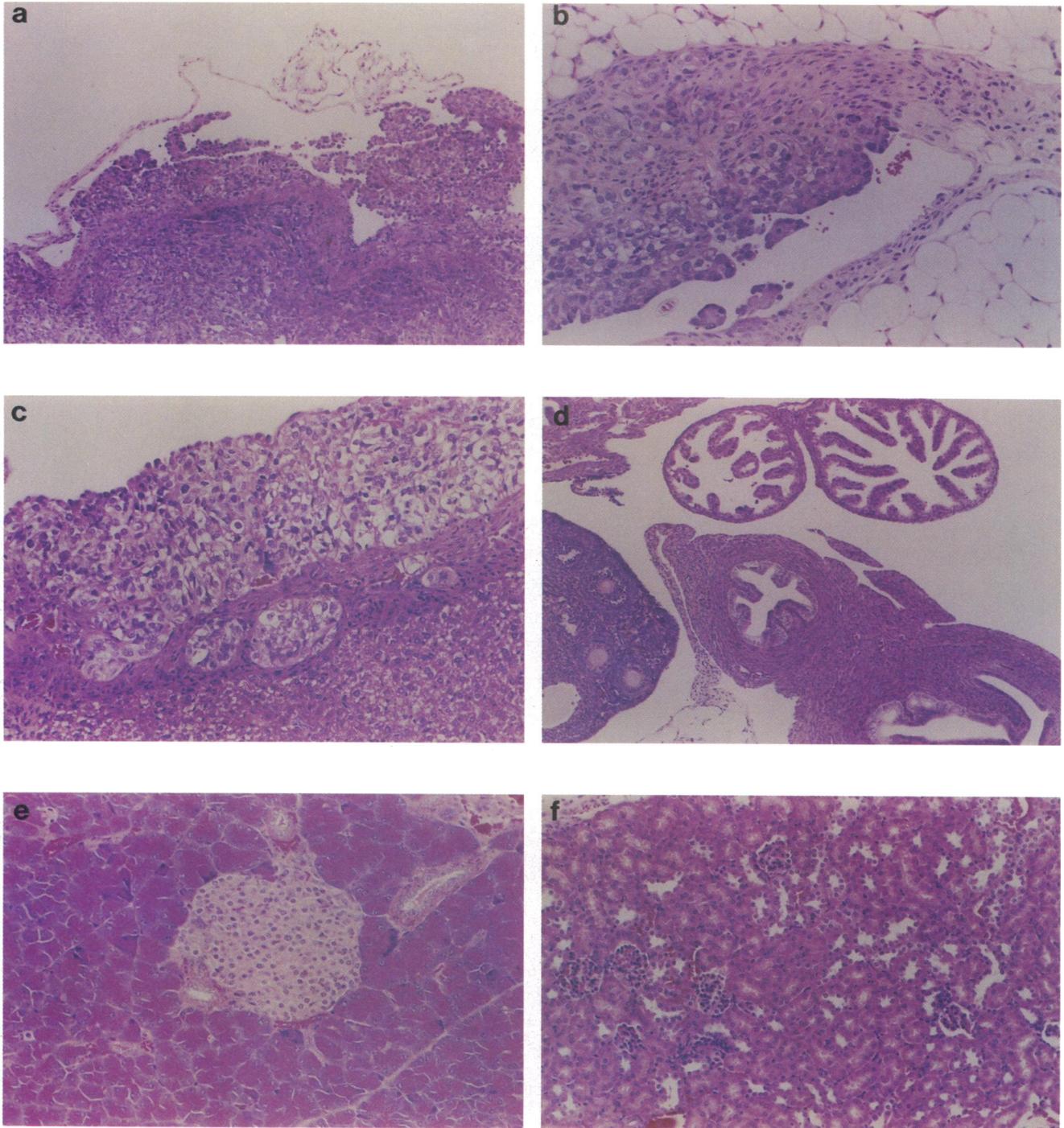


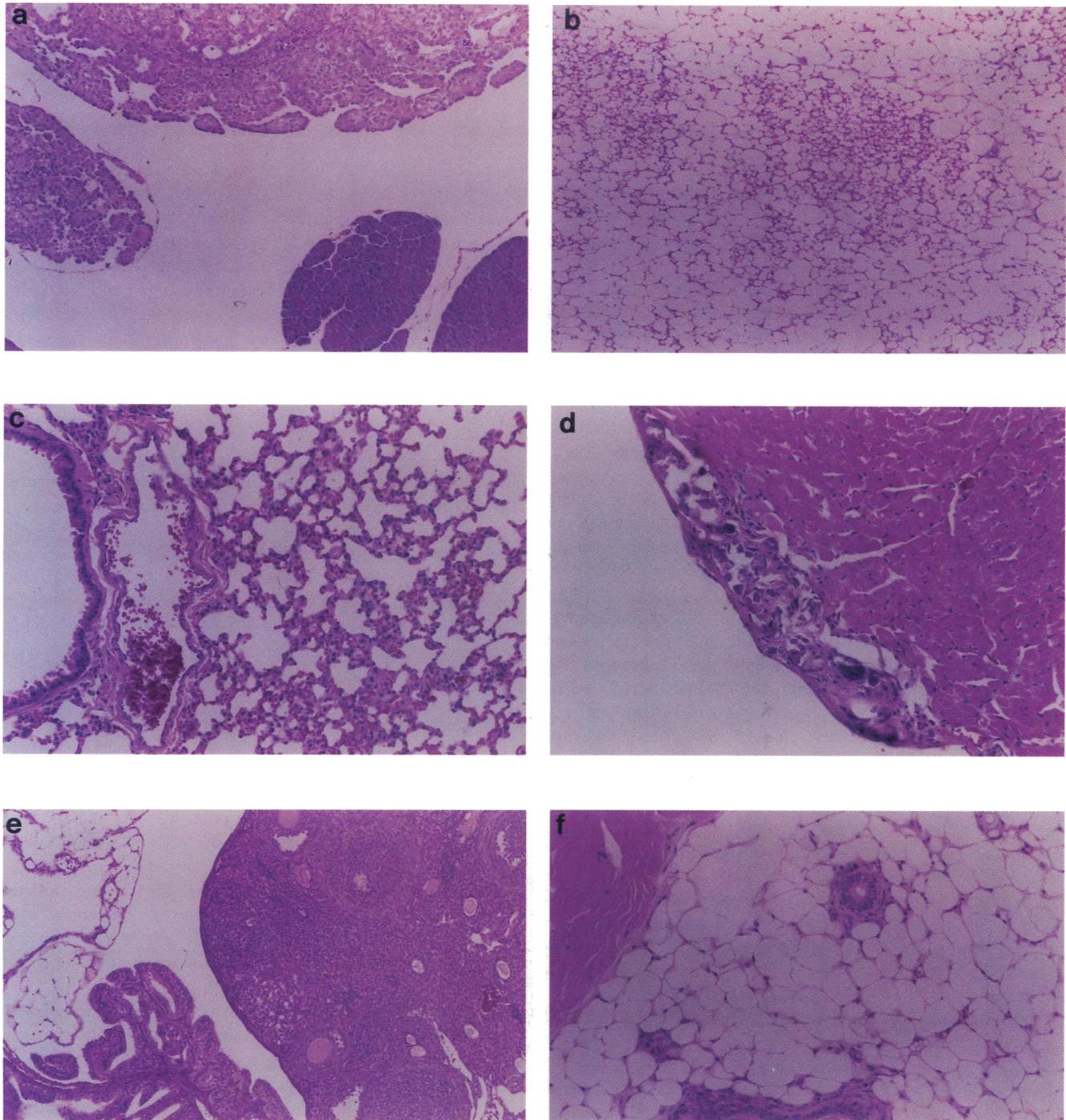
Figure 4 (Continued)

ceived only tumor cells and one additional group received only the recombinant adenovirus which served as an additional control group. Animals were assayed for survival. Statistical analysis was performed at day 36. Survival experience between control and experimental groups was compared, and statistical significance of difference was performed through the use of the log-rank test. To estimate the relative risks of

death comparing control groups with the experimental group, a proportional hazards model was used.

### Results

Advanced ovarian cancer is characterized by local dissemination of neoplastic cells within the peritoneal cavity. As most

**C***Figure 4 (Continued)*

patients first present with nonlocalized disease, gene therapy strategies must accomplish *in vivo* transduction of tumor cells *in situ* within the peritoneum. Previously proposed gene therapy protocols for carcinoma of the ovary have approached this problem by either intraperitoneal administration of an allogenic toxin gene encoding ovarian cancer cells or murine packaging cells expressing herpes simplex virus thymidine kinase (HSVtk)

encoding amphotropic retrovirus (18). We have proposed using a strategy to achieve selective cytotoxicity in *erbB-2*-expressing ovarian cancer cells via an intracellular anti-*erbB-2* single-chain antibody (sFv). This would be achieved by delivery of a gene construct encoding the anti-*erbB-2* sFv directly to tumor cells. The first step in developing this approach was to determine which vector system was capable of achieving the

highest frequency in situ transduction of ovarian cancer cells. In this regard, previous studies by Crystal (19) and Abelda (20) had demonstrated the utility of recombinant adenoviral vectors in accomplishing gene delivery to human tumor xenografts heterotopically transplanted into the peritoneum of athymic nude mice. In addition, our previous studies using the adenovirus-polylysine vector (AdpL) (13, 14) to accomplish intraperitoneal transduction of a lymphoblastoid cell line in a murine model of B cell lymphoma had demonstrated therapeutic efficacy of this vector (21). Preliminary in vitro studies had also suggested the utility of the cationic liposome vector DOTAP (11). We thus compared the relative efficacy of these vectors in the context of the gene transfer requirements of our strategy.

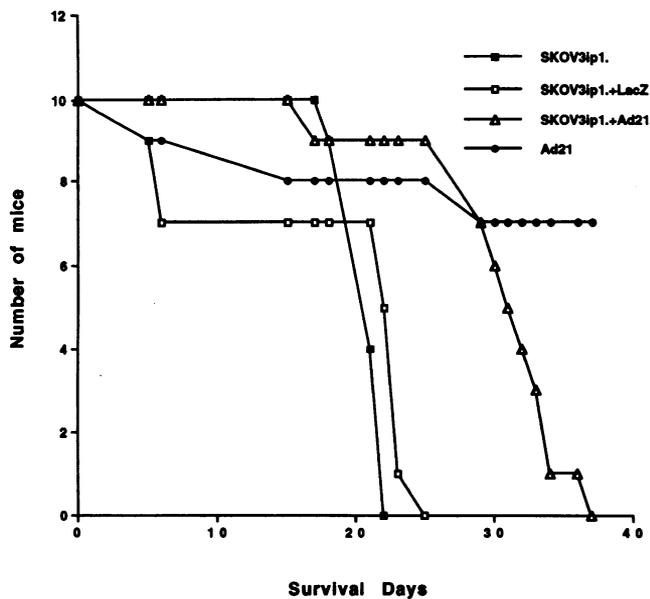
For this analysis, athymic nude mice were transplanted orthotopically with the human ovarian carcinoma cell line SKOV3. ip1 (22). Xenografting of this cell line had been shown to allow the establishment of a disease model with many of the characteristics of human ovarian carcinoma (22). In addition, this cell line is erbB-2 positive, allowing study of our sFv-mediated cytotoxicity strategy. The athymic nude mice were each transplanted with  $10^7$  SKOV3. ip1 cells via peritoneal inoculation. 5 d after transplantation, at which time malignant ascites had been established, the animals were challenged with the various vectors encoding an *E. coli*  $\beta$ -galactosidase reporter gene (LacZ). Animals thus received the LacZ-encoding adenovirus AdCMVLacZ ( $10^9$  pfu), AdpL-DNA complexes containing the LacZ-encoding plasmid CMV $\beta$  (2.0  $\mu$ g DNA), or DOTAP-DNA complexes containing the LacZ-encoding plasmid. 2 d after vector delivery, animals underwent peritoneal lavage to harvest mobile tumor cells for analysis of the erbB-2 oncoprotein and LacZ expression to indicate the specific subset of reporter gene-transduced tumor cells. In this analysis, the recombinant adenovirus accomplished transduction of > 80% of the malignant ascites tumor cell population (Fig. 1). This transduction frequency was significantly higher than that observed with the other evaluated vector systems. Based upon these results, a gene therapy strategy for ovarian cancer via anti-erbB-2 sFv gene transfer was designed using a recombinant adenovirus as the delivery vehicle.

We have shown previously that an ER-directed anti-erbB-2 sFv could accomplish specific cytotoxicity in erbB-2-overexpressing tumors of diverse histologic types (11). In addition, we have described previously the construction of a recombinant adenoviral vector encoding this gene construct (15). This vector is an E1A-deleted, replication-defective adenovirus derived by standard in vivo homologous recombination techniques. We next sought to demonstrate that the anti-erbB-2 sFv gene in this vector could function in a manner to achieve the desired tumor cell-specific cytotoxicity. For this analysis, tumor cells were treated in vitro with the adenovirus encoding the ER-directed anti-erbB-2 sFv (Ad21) or an adenovirus encoding the LacZ reporter gene (AdCMVLacZ). Treated cells included the erbB-2-overexpressing ovarian cancer cell line, SKOV3. ip1, as well as the non-erbB-2-expressing human cervical carcinoma cell line HeLa. Adenoviral vector-transduced cells were then examined for viability using the XTT assay as described previously (11, 15). In this analysis, it could be seen that the Ad21 vector accomplished a marked reduction in the number of viable SKOV3. ip1 tumor cells (Fig. 2). Using an moi of 50 particles per cell, > 95% of the SKOV3. ip1 tumor

cells were killed. Importantly, this cytotoxicity was selective; no decrement in viable cell number was noted in the non-erbB-2-expressing HeLa cell line treated with the Ad21 vector. This finding is consistent with the concept that the erbB-2 sFv is selectively cytotoxic in erbB-2-overexpressing tumor cells. This selective cytotoxicity is retained in the context of the anti-erbB-2 sFv encoding the adenoviral construct. Further in this regard, we have also shown that expression of the anti-erbB-2 sFv is nontoxic in a variety of erbB-2-negative human cells of diverse histologic type (data not shown). It is also noteworthy that the control reporter gene encoding adenovirus was noncytotoxic when delivered at the same moi, excluding the possibility of nonspecific vector-associated toxicity.

We next established the capacity of the adenoviral vector encoded anti-erbB-2 sFv to accomplish tumor cell killing in a disease-relevant context. For this analysis, the Ad21 vector was administered to animals which had been orthotopically engrafted with SKOV3. ip1 cells 5 d previously. 48 h after vector administration, mobile tumor cells were harvested by peritoneal lavage, and tumor cells were analyzed for the phenotypic effects of the anti-erbB-2 sFv gene transfer and expression. To determine the capacity of the adenoviral vector to accomplish in vivo tumor cell cytotoxicity, in situ lavage-harvested tumor cells were evaluated for cell viability using the XTT assay. In this analysis, it could be seen that the Ad21 vector was capable of marked reduction in the number of viable tumor cells compared with the control vector AdCMVLacZ (Fig. 3 A). Thus, in the context of mobile tumor cells in malignant ascites, the adenoviral vector was capable of achieving in situ transduction with selective tumor cell cytotoxicity. Further in this regard, previous in vitro studies had demonstrated that the basis of the sFv-mediated cytotoxicity was induced apoptosis (11). To determine if this was also the operative mechanism in vivo, harvested tumor cells were analyzed for phenotypic changes indicative of apoptosis. For this analysis, tumor cells from the treatment groups were analyzed for differential nuclear dye staining, a hallmark of cellular apoptosis (23). It was noted that the tumor cells harvested from the AdLacZ-treated animals were viable. In contrast, tumor cells harvested from the Ad21-treated animals exhibited dye staining characteristics consistent with induced apoptosis (Fig. 3 B). Thus, the anti-erbB-2 sFv-encoding adenoviral vector was capable of induced cytotoxicity in tumor cells based upon selective induction of the apoptosis program.

As a more stringent test of the efficacy of this approach, the ability of the Ad21 vector to reduce an established tumor burden was examined. In this analysis, SKOV3. ip1 tumor cells were established orthotopically as before (22), and animals were treated with the control adenovirus or the adenovirus encoding the anti-erbB-2 sFv. Animals were maintained for 14 d after treatment, and then harvested for a determination of tumor volume. Animals treated with the control adenoviral vector had extensive tumor burdens. Pathologic evaluation demonstrated large tumor nodules with marked invasion of abdominal viscera (liver, lung, and uterus) (Fig. 4, A and B). In contrast, animals treated with the Ad21 vector had dramatically decreased tumor burdens (Fig. 4 C) with a reduction in the number and size of residual tumor nests. Tumor cell invasion was not noted in this group. In addition, rare animals in this group were free of tumor by gross and microscopic evaluation. Thus, the



**Figure 5.** In vivo efficacy of the recombinant adenovirus encoding the ER form anti-erbB-2 sFv in prolongation of survival. erbB-2-overexpressing human ovarian carcinoma cells SKOV3. ip1 were injected intraperitoneally into CB-17 SCID mice ( $n = 10$ ). 5 d later the animals were challenged with AdCMVLacZ or Ad21. Animals were assayed for survival.

delivery of the anti-erbB-2 sFv by this vector approach resulted in a significant antitumor effect.

The ability of the Ad21 vector to modulate survival of ovarian cancer tumor-bearing animals was also evaluated. For these studies, SCID mice were xenografted with the SKOV3. ip1 cells as before. After the establishment of tumor, the animals were treated with either the control or anti-erbB-2 sFv adenovirus and evaluated for survival. Experimental groups included animals receiving tumor only, tumor followed by control adenovirus infection, or tumor followed by Ad21 infection. The results of this analysis are shown in Fig. 5. Using the log-rank test, significant differences in survival were observed among control groups ( $P < 0.01$ ), compared with the experimental group. The two control groups (cells only and cells plus AdCMVLacZ) had an increased risk of death by 12.4 and 6.4 times, respectively.

## Discussion

The overexpression of the erbB-2 tyrosine kinase receptor has been shown to be a key determinant in neoplastic transformation and progression in a variety of neoplasms including epithelial malignancies of the breast, lung, stomach, and ovary (24–30). Importantly, in carcinoma of the breast and ovary, the overexpression of erbB-2 correlates with a more aggressive tumor phenotype, indicative of a less favorable clinical outcome (26, 28). Based upon these concepts, many therapeutic approaches have been developed which target the erbB-2 oncoprotein (31). For example, specific antibody-based approaches have been explored using antitumor erbB-2 antibodies or anti-erbB-2 antibodies conjugated to specific antitumor moieties (32–42).

As an alternate approach to target the erbB-2 oncoprotein,

strategies have been developed to directly modulate the levels of the overexpressed tyrosine kinase receptor. These approaches have included triplex-forming oligonucleotides to achieve transcription block, as well as antisense oligonucleotides to target posttranscriptional erbB-2 transcript species (5, 43, 44). The utility of these approaches has been partially characterized in various in vitro systems. In these studies, it could be shown that downmodulation of cell surface erbB-2 may be achieved with an effect of proliferation inhibition (32, 38, 39, 41, 43, 44). As an alternate means to achieve knockout of the erbB-2 oncoprotein, we have developed a strategy based upon intracellular expression of the anti-erbB-2 single chain antibody (10). In addition, others have also used the intracellular antibody strategy to achieve functional ablation of the erbB-2 oncoprotein (45, 46). We have demonstrated that this approach can achieve cytotoxicity in erbB-2-overexpressing human cells (11, 12). It appears that this effect is achieved by virtue of sFv-mediated erbB-2 mislocalization inducing cellular apoptosis (11). Importantly, this antitumor cytotoxicity is targeted in that non-erbB-2-expressing cells do not appear susceptible to the deleterious effects of intracellular anti-erbB-2 sFv expression.

We have explored the potential of this approach as a gene therapy strategy for ovarian carcinoma. In this regard, erbB-2-positive ovarian primary tumor material demonstrated the same sensitivity to anti-erbB-2 sFv mediated cytotoxicity as we had observed previously in ovarian cancer cell lines (12). Based upon these findings, we have explored the efficacy of this approach in animal models of human ovarian cancer in this report. Our studies demonstrate that adenoviral-mediated delivery of the anti-erbB-2 sFv can accomplish an antitumor effect with a demonstrated survival advantage in treated animals.

One attractive feature of this approach is that the intracellular expression of the anti-erbB-2 sFv is selectively toxic for erbB-2-overexpressing tumors. Thus, the mandate to achieve tumor cell-specific transduction is obviated, as ectopic expression of the anti-erbB-2 sFv, even if it were to occur, is not deleterious. Additionally, in ovarian cancer, where containment of intraperitoneally delivered vector is desired, ectopic expression of the therapeutic gene construct is not a major limitation. Indeed, this has been recognized in other anti-ovarian cancer gene therapies (19, 20), whereby vector confinement within the peritoneal cavity has been demonstrated. However, it must be noted that these later proposals are based upon selective delivery of the HSVtk gene. Ectopic Tk gene expression could not be considered without consequence, as its mechanism of cytotoxicity is less dependent upon tumor-specific factors, as is the case within the intracellular antibody strategy.

One potential limitation of this strategy is the requirement to accomplish transduction of all neoplastic cells to achieve tumor eradication. Despite this consideration, we show that a nonoptimized vector delivery dose and schedule achieved tumor reduction at a level capable of accomplishing survival prolongation. These issues will nonetheless be key determinants in the employment of this strategy in the human setting. Thus, it is unclear if antivector immunity will limit the ability to use this vector in repetitive delivery (47). Studies are currently underway to evaluate this issue in other adenoviral delivery schemas. However, it must be noted that vector transduction of all tumor targets may be a theoretical requirement only. In this regard, a greater efficacy of HSVtk gene therapy has been noted in immunocompetent as compared with immunodeficient animals.

It has been postulated that the Tk-killed tumor fraction functions as an effective vaccine, allowing the enhanced development of an antitumor immunity (48). Further in this regard, Baserga (49) has shown recently that tumor cells treated to achieve IGF-1-R knockout function as highly effective antitumor vaccines capable of accomplishing eradication of established syngeneic tumor in rodent models. These studies suggest that apoptotic cells may elicit better antitumor immunization. Considered in this context, the present strategy may likewise allow for antitumor events over and above direct sFv-mediated tumor cell killing.

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