The Role of Natural Killer Cells in Adenovirus-mediated p53 Gene Therapy

Jennifer L. Carroll, Loretta L. Nielsen, Stephen B. Pruett, and J. Michael Mathis

Departments of Cellular Biology and Anatomy [J. L. C., S. B. P., J. M. M.] and Obstetrics and Gynecology [J. M. M.], Feist-Weiller Cancer Center [J. L. C., J. M. M.], Louisiana State University Health Sciences Center, Shreveport, Louisiana 71130, and Division of Tumor Biology, Schering-Plough Research Institute, Kenilworth, New Jersey 77033 [L. L. N.]

Abstract

Adenovirus-mediated gene therapy is a promising new approach for treatment of ovarian cancer. In animal models, complete elimination of cancer cells is often achieved, although the therapeutic gene has not been delivered to all these cells. This is referred to as a bystander effect, because tumor cells near those that receive the therapeutic gene are also eliminated. Several mechanisms have been proposed for the bystander effect, including intercellular communication within the tumor via gap junctions, apoptosis, antiangiogenesis, cytokines or other soluble mediators, and immunological mechanisms. There are two well-documented antitumor effector cell populations in athymic nude mice: macrophages and natural killer (NK) cells. We hypothesize that peritoneal populations of NK cells in nude mice treated with adenoviruses are involved in the observed bystander effect in this in vivo model. We investigated the role of NK cells as immunological mediators for the bystander effect using the p53 tumor suppressor as the therapeutic anticancer gene. Most ovarian cancer cell lines tested were sensitive to lysis by NK cells, although different ovarian cancer cell lines exhibited different sensitivities to NK cell-mediated lysis. To determine the importance of NK cells in the overall efficacy and in the bystander effect of gene therapy, NK cells were depleted in mice by administration of anti-NK1.1 monoclonal antibodies. To study the efficacy of NK depletion, C57BL/6 (nu/nu) mice were given injections i.v. by a single tail vein injection or i.p. with increasing doses of anti-NK1.1 IgG. All doses of anti-NK1.1 antibody, from 100–500 μg, essentially eliminated cytotoxic NK activity. To assess the duration of depletion after a single dose of anti-NK1.1 IgG, a time-course experiment was performed. NK 1.1 antibody was effective in completely depleting cytotoxic NK cell activity in the mice for up to 7 days, whether given as 500 μg (i.p.) or 200 μg (i.v.). Flow cytometric analysis performed on peritoneal cell populations confirmed depletion of NK cells by ~80%. Finally, a survival study was performed, in which animals were depleted of NK cells. In this experiment, NK cell-depleted mice were injected with anti-NK1.1 IgG, and control mice were mice were treated with normal saline. Two days later, all mice were inoculated with a lethal i.p. dose of NIH:OVCAR-3 ovarian cancer cells. After 3 days, the mice were divided into two treatment groups; one treatment group received three consecutive daily i.p. injections of Ad-CMV-p53 (SCH58500), and the second treatment group received three consecutive daily i.p. injections of control adenovirus construct, rAd-null. All of the NK cell-depleted animals, whether treated with rAd-null or with Ad-CMV-p53 (SCH58500) were dead of disease by 116 and 138 days, respectively, after initiation of adenovirus treatment, and no statistically significant difference in survival was observed (P = 0.349). A significant survival advantage was seen in control (NK-competent) mice treated with rAd-null (P = 0.04), although all were dead of disease by day 184. Importantly, control NK-competent mice treated with Ad-CMV-p53 (SCH58500) showed no tumor growth or ascites production, and all animals survived. These results indicate that immunological mechanisms involving natural killer cells play an important role in the bystander effect involving adenovirus-p53 gene therapy for ovarian cancer.

Introduction

Ovarian cancer arises from the accumulation of mutations in multiple combinations of genes (1). These changes involve the inherited or acquired activation of cellular proto-oncogenes and somatic or germ-line inactivation of tumor suppressor genes. The most extensively studied tumor suppressor gene in solid tumors is p53, a Mₚ, 53,000 nuclear phosphoprotein that binds DNA. The p53 gene product plays a role in normal cellular proliferation by regulating gene transcription, cell cycle control, and apoptosis (2). Mutations of p53 are the most common molecular genetic abnormality to be described in human cancer and have been identified in malignancies of the breast, colon, lung, esophagus, head and neck, and hematopoietic system (3). Mutations of the p53 gene have been identified in 30–79% of epithelial ovarian cancers (4, 5). Most of the mutations identified in p53 are distributed throughout the open reading frame as missense mutations.
Despite cancer’s polygenic nature, there is strong evidence that correction of only one of the genetic defects in cancer cells in vitro can lead to the inhibition of cell growth and reversal of tumorigenicity (5–7). The strategy of human gene therapy to alter the biology of human cancers is an area of investigation that shows significant potential for improving survival (8). Functional impairment of p53 resulting from gene deletion or mutation is associated with the loss of cellular checkpoint signaling functions and the inability to coordinate programmed cell death (apoptosis) in the presence of DNA damage (9). Even in a background of genetic heterogeneity, replacement of a single wild-type p53 allele can be effective in reversing the malignant phenotype (6, 10). Because this gene is frequently mutated in human cancers, it has emerged as a promising target of gene therapy techniques.

The E1-deleted, type 5 adenovirus-based vector has emerged as a leading candidate of in vivo gene therapy (11). The adenoviral vector can be produced in high titers, does not integrate into the host chromosome, and has a wide tropism (12). In addition, adenoviral vectors infect both dividing and nondividing cells with great efficiency and have nearly 100% transfection efficiency, but the expression of the transferred gene is transient, because the viral genome remains episomal. However, adenoviruses are limited by hepatotoxicity at high adenovirus doses in vivo (13, 14). We have shown that adenovirus-mediated transfection of wild-type p53 into p53-mutated ovarian cancer cells is feasible and results in growth inhibition in vitro (15–18). The adenovirus vector transfects p53 efficiently, allows transient expression of the p53 protein product, and results in induction of apoptosis. The immunodeficient mouse is an established animal model system to study i.p. dissemination in ovarian cancer (19, 20). Human ovarian cancer cell lines grow in the peritoneal cavity of T-cell deficient (nu/nu) mice and induce ascites and intra-abdominal carcinomatosis.

Using an in vivo mouse model system, the i.p. route of administration of the adenovirus vector is an appropriate approach for the treatment of ovarian cancer metastasis, inasmuch as i.v. administration is much less efficient (21). Ovarian cancer remains confined to the peritoneal cavity for the majority of its course (22). Therefore, therapeutic agents can be easily and effectively delivered to the tumor with pharmacokinetics that are often better than systemic administration. Thus, the i.p. localization of epithelial ovarian cancer makes this disease particularly amenable to gene therapy strategies, and understanding these interactions will allow us to identify patients more likely to benefit from clinical trials using i.p. administration of adenovirus-mediated p53 gene therapy.

In previous studies, we characterized the efficacy of adenovirus-based p53 gene therapy in the treatment of ovarian cancer using a microscopic i.p. disease animal model system (15). Our in vivo studies using the athymic (nu/nu) mouse as a model for i.p. cancer has demonstrated a survival advantage in animals treated with this agent (16). The mechanism of wild-type p53 growth inhibition in tumor cells is complex and may involve cell cycle control through induction of target genes, or it may involve induction of programmed cell death or apoptosis. Death of tumor cells modified with the wild-type p53 gene may also lead to the killing of uninfected neighboring tumor cells in a phenomenon termed the bystander effect (23–27).

Preclinical p53-based gene therapy has been shown to be effective against cancer both in vitro and in vivo (28–31). Early clinical studies of p53-based gene therapy also show promise (32, 33). The ability of an adenovirus to infect 100% of cells within a tumor in vivo is limited, because tumor bulk and size may limit viral infectivity. In our studies, however, the treatment of athymic mice harboring human ovarian tumors with an adenovirus carrying the wild-type p53 gene caused complete inhibition of tumor formation (15, 16). These results suggest the mechanism of killing by the Ad-CMV-p533 (SCH58500) involves a bystander effect, whereby uninjured neighboring cells are killed by a factor or signal from the infected cells.

There are two well-documented antitumor effector cell populations in athymic nude mice: macrophages and NK cells. We hypothesize that peritoneal populations of NK cells in nude mice treated with adenoviruses are involved in the observed bystander effect. The results of this study suggest that the mechanism of killing by the Ad-CMV-p53 (SCH58500) does involve a bystander effect, in which the in vivo treatment of ovarian cancer with Ad-CMV-p53 (SCH58500) results in NK cell activation and recruitment to the peritoneal cavity.

Materials and Methods

Cell Culture and Cell Lines. The NIH:OVCAR-3 and YAC-1 cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured as recommended. NIH:OVCAR-3 cells were maintained in DMEM:Ham’s F-12 (Life Technologies, Inc., Rockville, MD) containing 20% FBS (Gemini Bioproducts, Calabasas, CA), and insulin-transferrin-selenium supplement (Life Technologies, Inc., Rockville, MD). YAC-1 cells were maintained in RPMI 1640 with l-glutamine containing 10% FBS. The cells were maintained in T-75 flasks at 37°C and 5% CO2 and subcultured using 1% trypsin-EDTA. The 222, 2774, PA-1, SKOV-3, UCI 101, and UCI 107 cell lines were cultured as described previously (34) in DMEM:Ham’s F-12 medium containing 10% FBS.

Adenoviruses. The replication-defective human adenovirus (serotype 5-derived) vectors were produced in 293 cells via homologous recombination between two transfected plasmids containing adenovirus DNA fragments overlapped at the E1a flanking region. Construction of the Ad-CMV-p53 virus (SCH58500; Schering-Plough Research Institute, Kenilworth, NJ) has been described previously (35). The control rAd-null construct, consisting of an E1a-deleted adenovirus with no CMV promoter and no transgene cassette, was produced by Canji, Inc. (ZZNB; San Diego, CA). The concentration of total viral particle numbers was determined by measuring absorption at 260 nm (36). Infectious particle

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3 The abbreviations used are: CMV, cytomegalovirus; C.I.U., cellular infectious units; NK, natural killer; FBS, fetal bovine serum; FACS, fluorescence-activated cell sorter; poly I:C, polyinosinic:polycytidylic acid; HSV-tk, herpes simplex virus thymidine kinase; IL, interleukin; LAK, lymphokine-activated killer cells.
numbers were determined by measuring the concentration of viral hexon protein-positive 293 cells after a 48-h infection period (37).

NK Cell Depletion. Female C57/BL6 (nu/nu) mice (Jackson Labs, Bar Harbor, ME, or Taconic, Germantown, NY) were depleted of NK cell activity by administration of a rabbit anti-NK1.1 IgG antibody (PK136) that efficiently depletes NK cells in vivo (38). Mice were given injections either i.p. or i.v. via tail-vein injection of 100–500 μg of antibody diluted to 0.5 ml in normal saline. As a control, mice were given injections of an equivalent dose of a nonspecific isotype control antibody (rabbit IgG2a-κ; Zymed Laboratories, San Francisco, CA).

Peritoneal and Splenic NK Cell Isolation. Animals were killed by CO2 asphyxiation, and the skin was removed from the peritoneal surface. Seven ml of ice-cold PBS containing 2% FBS were injected into the peritoneal cavity below the liver. A vigorous external peritoneal massage was performed to dislodge cells. Using great care not to cause bleeding, the peritoneal lavage fluid was withdrawn and collected in sterile tubes. The fluid was centrifuged at 1000 rpm for 7 min. The cell pellet was resuspended in 1 ml of complete medium (RPMI 1640 with 10% FBS and 1% penicillin-streptomycin). Ten μl of cell suspension were counted on a hemacytometer or automatic cell counter. Peritoneal cell samples were adjusted to 1 × 10⁷ cells/ml. Spleens were harvested and homogenized manually between the ends of frosted glass microscope slides. Dispersed splenic cells were centrifuged at 1000 rpm for 5 min and resuspended in 3 ml of complete medium. Cell suspensions were counted using an automatic cell counter and adjusted to 1 × 10⁷ cells/ml.

Cytospin Preparations and Immunostaining. Fifteen-μl aliquots of the peritoneal suspension were used for cytospin preparations on slides. The slides were centrifuged at 1000 rpm for 5 min. Slides were air-dried overnight and either stained with a leukocyte stain kit (LeukoStat stain system; Fisher Scientific, Pittsburgh, PA) or fixed in 95% ethanol for 30 s. Fixed slides were processed for immunocytochemistry using antibodies directed against B cells (rat anti-B220; Caltag Laboratories, San Francisco, CA), macrophages (rat anti-F480; Caltag Laboratories), NK cells (rat anti-pan NK cell; Pharmingen; San Diego, CA), and the α/β T cell receptor (hamster anti-α/β TCR; Caltag Laboratories). A 3,3′-diaminobenzidine kit was used to detect the presence of the above listed cell types (BioGenex Laboratories, San Ramon, CA). Quantitation by digital imaging microscopy revealed NK cells as 1.9% of the total peritoneal cell population. The B cells accounted for 0.2% and 0.5%, respectively.

Flow Cytometric Analysis of Peritoneal and Splenic Cells. One hundred μl of cells (1 × 10⁷ cells/ml) were added to a number of wells in 96-well “V”-bottomed plates. The cells were centrifuged at 1200 rpm for 4 min and then resuspended in 200 μl of cold FACS buffer [0.1% BSA and 0.1% sodium azide in PBS (pH 7.4)]. All wells were treated with 2 μl of anti-Fc receptor antibody (Pharmingen), except those to be stained for macrophages (which received 5 μl), to block the nonspecific binding of antibodies (via Fc receptors) to splenic and peritoneal cells. The plates were incubated at 4°C for 15 min. Antibodies against the following four cell types were used: B cells (rat anti-B220-FITC; Caltag Laboratories); macrophages (rat anti-F480-PE; Caltag Laboratories); NK cells (rat Pan anti-NK cell-PE; Pharmingen); and the α/β T cell receptor (hamster anti-TCR-FITC; Caltag Laboratories). Isotype antibodies were also used to set up compensation and quadrants for the flow cytometric analysis. Antibodies against the cells of interest were added at the appropriate concentrations (3 μl for B cells and macrophages, 2 μl for NK cells, and 5 μl for T cells). The plates were incubated at 4°C in the dark for 30 min. After centrifuging the plate at 1200 rpm for 4 min, the cells were resuspended in 100 μl of 37°C RBC lysis buffer and then incubated for 5 min at 37°C. The plates were centrifuged, and the cells were resuspended in cold FACS buffer. The plates were then centrifuged, and the cells were resuspended in 100 μl of 4°C paraformaldehyde and incubated for 5 min in the dark. The plates were centrifuged, and the cells were resuspended in 200 μl of FACS buffer. The cells were analyzed using a FACScan flow cytometer (Becton Dickinson; Franklin Lakes, NJ).

Chromium Release Assays. To measure sensitivity of human ovarian cancer cells to mouse NK cell lysis, a chromium release assay was performed. Ovarian cancer cell lines 222, 2774, OVCAR-3, PA-1, SKOV-3, UCI 101, and UCI 107 were labeled with ⁵¹Cr after a protocol modified from Lucci et al. (39). The cells were harvested from T-75 flasks, counted, and resuspended at a concentration of 1 × 10⁶ cells/10 ml of medium. To each cell suspension, 125 μCi of ⁵¹Cr (ICN Pharmaceuticals, Costa Mesa, CA) were added as an aqueous solution of sodium chromate. The cells were then plated out in various concentrations (for specific E:T ratios) in quadruplicate in two flat-bottomed, 96-well plates. One plate was used for the chromium release assay and the other plate was used to determine the number of cells present at the time of the assay, so that a consistent E:T ratio could be maintained. The cells in the control plate were trypsinized and counted on a hemacytometer. Appropriate adjustments were made with splenic and peritoneal cell numbers added to the ⁵¹Cr plate to ensure E:T ratios. The entire assay was carried out under sterile conditions. YAC-1 (NK-sensitive) and P815 (NK-insensitive) cells were used as control target cells for this assay. One hundred μl of supernatant were harvested at 4 h after the addition of effector cell, and the remaining 100 μl were harvested at 24 h for analysis on a gamma counter. The percentage of specific lysis was determined for each E:T ratio and cell line at both time points. Two time points were used to ensure lysis of the target cells (ovarian cancer cells).

To measure NK cell activity in vivo, a chromium release assay was used on harvested peritoneal or splenic cells. One day before ⁵¹Cr assay, control or experimental animals were treated with 100 μg of poly I:C (Sigma Chemical Co., St. Louis, MO). On the day of assay, YAC-1 target cells were adjusted to 1 × 10⁷ cells/ml in saline and incubated with 500 μCi of ⁵¹Cr (Na²⁶⁵CrO₄; ICN Pharmaceuticals) for 90 min at 37°C, swirling every 15 min. The labeled target cells were washed once in HBSS (Sigma Chemical Co.) and three times in RPMI 1640 (Life Technologies,
NK Cells in Adenovirus-mediated p53 Gene Therapy

Inc.) and then adjusted to a concentration of $1 \times 10^6$ cells/ml. The isolated peritoneal cells and splenocytes were added to wells of a U-bottomed, 96-well plate (50, 25, and 12.5 $\mu$l for each sample). All wells with cells were brought to a final volume of 100 $\mu$l with complete medium (RPMI 1640 with l-glutamine containing 10% FBS). Two sets of controls were used. Six wells contained only 100 $\mu$l of complete medium to account for spontaneous release, and six wells contained 100 $\mu$l of 2 $\times$ HCl to account for maximum release of $^{51}$Cr. One hundred $\mu$l of the labeled target cells were added quickly to each of the wells on the plates. The plates were incubated at 37°C for 4 h. After incubation, the plate was centrifuged at 1200 rpm for 5 min to pellet the cells. One hundred $\mu$l of the resulting supernatant was removed to glass tubes for analysis on a gamma counter.

**NK Cell Activation by Adenovirus.** On day 1, animals received $1 \times 10^8$ freshly harvested NIH:OVCAR-3 human ovarian cancer cells in 1 ml PBS by i.p. administration. Adenoviral treatments were carried out on days 4–6. Animals received a daily i.p. injection of $1 \times 10^9$ viral particles of Ad-CMV-p53 (SCH58500), a 10:1 virus:tumor cell dose that resulted in 10–50% growth inhibition in vitro (16). Control animals received 1 ml of normal saline. Animals were sacrificed 2 days before, 1 day after the completion of, or 7 days after the completion of adenovirus treatment. Chromium release assays were performed as described above on harvested peritoneal and splenic NK cells.

**NK Cell Depletion in Vivo.** To study the efficacy of NK depletion, animals were given injections i.p. with 500 $\mu$g or i.v. by a single tail vein injection with 100–200 $\mu$g of anti-NK1.1 IgG. Twenty-four h later, the mice were injected i.p. with 100 $\mu$g of the NK-activating substance, poly I:C. On the next day, the cytotoxic activity of peritoneal cells was assessed in a chromium release assay against NK-sensitive YAC-1 target cells.

**Survival Studies.** On day 1, female C57/BLE6 (nu/nu) mice (Jackson Labs or Taconic) received 500 $\mu$g as an i.p. injection of NK1.1-depleting antibody (PK136) or isotype control antibody, IgG2a (Zymed Laboratories). On day 3, the mice received an i.p. injection of $1 \times 10^9$ freshly harvested NIH:OVCAR-3 cells in 1 ml of saline. On days 6–8, animals received daily i.p. injections of $1 \times 10^9$ viral particles, either Ad-CMV-p53 (SCH58500) or rAd-null. The adenovirus vectors were diluted to $1 \times 10^9$ C.I.U./ml with PBS, drawn into syringes, and administered into the peritoneal cavity of animals using a 20-gauge needle within 15 min of dilution. On day 9, and at weekly intervals for 1 month, animals received doses of anit-NK1.1 antibody or isotype control antibody (500 $\mu$g i.p. in 0.5 ml). The animals were monitored daily for changes in health, including overall appearance (i.e., weight changes, signs of inflammation, or ulceration) and activity. Animals with tumors were monitored by gross examination and palpation for the presence of lesions that represented excessive size (>10% of body weight) or lesions that impaired mobility. Animals that exhibited significant tumor burden, weight loss, or pain and distress were killed by asphyxiation with CO$_2$ according to the guidelines of the Institutional Animal Care and Research Advisory Committee at Louisiana State University Health Sciences Center-Shreveport, Shreveport, LA. Peritoneal lavages were performed, and har-vested cells were cultured to determine the presence of viable OVCAR-3 cells. A necropsy was performed on each animal to determine the gross extent and distribution of tumor burden. Peritoneal organs were removed and fixed in 10% formalin. The tissues were paraffin-embedded, and sections were cut and stained with H&E and examined by light microscopy. Survival was analyzed by the Kaplan-Meier method, and comparisons between treatment arms were made by the log-rank test. Statistical significance was based on a $P$ of $<0.05$. Pretreatment and posttreatment effects in each group were evaluated by the paired Student $t$ test.

**Results**

**Assessment of NK Cell Activity.** To determine the sensitivity of ovarian cancer cell lines as targets for NK cell lysis, a chromium release assay was performed on seven ovarian cancer cell lines (222, 2774, OVCAR-3, PA-1, SKOV-3, UCI 101, and UCI 107). As controls, a NK-sensitive cell line (YAC-1 mouse lymphoma cells) and an NK-insensitive cell line (P815 mastocytoma cells) were also assayed. As shown in Fig. 1, all human ovarian cancer cell lines that we examined were sensitive to lysis by NK cells. Although the ovarian cancer cell lines showed different sensitivities to NK cell-mediated lysis, NIH:OVCAR-3 cells showed a similar NK sensitivity profile to that of YAC-1 target cells. In the 4-h incubation with P815 (NK-insensitive) target cells used in this assay, very little or no cytolytic activity was detected. The P815 cells, however, are targets of macrophage cell lysis, although to detect cytolytic activity represented by macrophages, much longer 16–24 h incubations are necessary (40). Thus, although NK cells were not purified, they represent the only cell population capable of lysing the effector cells is this assay.

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**Fig. 1.** Chromium release assay of ovarian cancer target cell lines. Seven human ovarian cancer cell lines (222, 2774, OVCAR-3, PA-1, SK-OV-3, UCI-101, SK-OV-3, and UCI-107) were tested for sensitivity to lysis by mouse NK cells and compared with the sensitivity of the YAC-1 mouse lymphoma cell line (sensitive to NK lysis, ○) and to the P815 mastocytoma cell line (insensitive to NK lysis, □). Results shown are the mean ± SE for triplicate cultures.
peritoneal lavages. Animals were killed, and NK-positive staining cells were determined in

Treatment with Ad-CMV-p53 (SCH58500), however, activated peritoneal NK cells in vivo for a period of between 1 and 7 days (Fig. 3, B and C). In subsequent studies, rAd-null was used as a control for any specific adenoviral activation effects and, as shown in Fig. 7, produced a NK cell activation profile similar to Ad-CMV-p53 (SCH58500).

**NK Cell Depletion.** To study the efficacy of NK depletion, mice were given injections either i.p. or i.v. by a single tail vein injection with anti-NK1.1 IgG. As shown in Fig. 4, peritoneal cells from mice injected with poly I:C alone showed significantly elevated cytolytic activity compared with peritoneal cells from control (PBS-treated) mice. All doses of anti-NK1.1 antibody, delivered either i.p. or i.v., efficiently reduced poly I:C stimulation of cytotoxic peritoneal NK cells compared with control (naïve or saline-treated) levels. In addition, treatment of animals with an isotype-control antibody had no effect on the reduction of NK cell activity; these animals had a similar NK cell activation profile to that of saline-treated animals (data not shown). These results demonstrate that either the i.p. or i.v. route of antibody administration are equally effective in depleting NK cells.

To assess the duration of depletion after a single dose of anti-NK1.1 IgG, a time-course experiment was performed. In the experiment shown in Fig. 5, mice were given injections i.v. with 200 μg or i.p. with 500 μg of anti-NK1.1 IgG. After 24 h, the mice were injected i.p. with 100 μg of poly I:C to activate NK cells. Isotype-control antibody experiments were also performed to ensure that there were no inhibitory effects of antibody treatment on NK cell activation or detection of that activation; isotype antibody-treated animals had an NK-activation profile similar to naïve animals (data not shown). These results demonstrate that the anti-NK1.1 antibody treatment was effective in completely reducing poly I:C stimulation of cytotoxic NK activity to control (naïve) levels for up to 7 days, whether given by i.p. or by i.v. administration.

Flow cytometric analysis was performed on peritoneal cell populations to confirm the depletion of NK cells by the anti-NK1.1 antibody (Fig. 6) and to monitor populations of B cells, T-cells, and macrophages (data not shown). Using a pan-NK cell antibody (which does not bind to the same antigen as the NK1.1 antibody) to detect cells, peritoneal cells from animals treated with control IgG or treated with anti-NK1.1 antibody were compared for numbers of NK cells after inoculation with OVCAR-3 cells. Control antibody-treated animals had an increased number of NK-positive cells compared with anti-NK1.1 antibody-treated animals (Fig. 6, C and G). And as expected, OVCAR-3 cells did not significantly increase the number of peritoneal NK cells in either treatment group (Fig. 6, D and H). In addition, administration of either rAd-null or Ad-CMV-p53 (SCH58500) resulted in the recruitment of NK cells to the peritoneum, compared with saline-treated animals or animals injected with OVCAR-3 cells alone (Fig. 6, E and F). Importantly, rAd-null administration recruited NK cells at a similar level to Ad-CMV-p53 (SCH58500) in the peritoneal cavities of the mice.
In repeated experiments, there was a small statistical difference between rAd-null and Ad-CMV-p53 (SCH58500) in the extent of NK cell recruitment (18.6 ± 0.7% versus 23.7 ± 2.8%). This result may be attributable to intrinsic differences in virus preparations or viral titers or possibly to a difference caused by transgene expression with Ad-CMV-p53 (SCH58500). Importantly, these results demonstrate that treatment with the NK1.1 antibody was effective in depleting NK cells by ~80% after adenoviral treatment when given as a 500-µg i.p. dose. However, adenovirus treatment did not alter peritoneal populations of B cells, T cells, and macrophages (data not shown). These results are consistent with previously published characterizations of the antibody in vivo (40). In addition, control animals treated with isotype-control antibody had similar numbers of peritoneal NK cells to that of saline-treated animals (data not shown).

In the experiments shown in Figs. 5 and 6, the animals were injected i.p. with the NK-activating substance, poly I:C. We observed in these experiments that doses of anti-NK1.1 antibody resulted in a very dramatic effect to reduce the poly I:C stimulation of cytotoxic peritoneal NK cells to levels comparable with that of control (naïve or saline-treated) activity. Likewise, by flow cytometric analysis, treatment with the NK1.1 antibody was effective in depleting NK cells by ~80%. These numbers of peritoneal NK cells were similar to those of control (saline-treated) animals. Together, these results demonstrate the efficacy and specificity of adenoviruses in the recruitment of NK cells to the peritoneum. In addition, these results confirm that NK cells can be depleted in mice for an extended duration.

Flow cytometry results reported in Fig. 6 showed a similar recruitment of NK cells by rAd-null administration and by Ad-CMV-p53 (SCH58500) administration. To determine...
whether rAd-null treatment alone could also activate peritoneal NK cells, a chromium release assay was performed. In this experiment, two animals received a single injection of $1 \times 10^9$ C.I.U. Ad-CMV-p53 (SCH58500), and two animals received a single injection of $1 \times 10^8$ C.I.U. rAd-null, and they were compared with control (saline-treated) mice or to mice treated with poly I:C. As shown in Fig. 7, peritoneal cells from mice injected with poly I:C alone showed significantly elevated cytolytic activity against YAC-1 target cells, compared with peritoneal cells from control (PBS-treated) mice. These results demonstrate that inoculation with either rAd-null or Ad-CMV-p53 (SCH58500) results in a similar NK cell-activation profile to that of poly I:C. Treatment of animals at higher doses of $10^{10}$ C.I.U. of rAd-null or Ad-CMV-p53 (SCH58500) resulted in similar levels of NK cell activation (data not shown). Thus, at the doses used in this study, we are likely at a maximal level of recruitment and activation of NK cells.

Survival Studies in Mouse Model of Human Ovarian Cancer. A survival study was performed in which animals were depleted of NK cells. In the experiment shown in Fig. 8, on the first day of the experimental protocol, 14 mice were treated either with anti-NK1.1 IgG or with normal saline. On day 3, all mice were inoculated with NIH:OVCAR-3 cells (i.p.). Beginning on day 6, the mice were divided into two treatment groups: one treatment group received i.p. injections of Ad-CMV-p53 (SCH58500), whereas a second treatment group received i.p. injections of rAd-null. All of the NK-depleted animals, whether treated with rAd-null or with Ad-CMV-p53 (SCH58500) were dead of disease by 116 and 138 days, respectively, after initiation of adenovirus treatment, and no statistically significant difference in survival was observed between the two viral treatments ($P = 0.349$). A significant survival advantage was seen in control (NK-competent) mice treated with rAd-null [$P = 0.04$ versus NK-depleted + rAd-null; $P = 0.02$ versus NK depleted + Ad-CMV-p53 (SCH58500)], although all animals were dead of disease by day 184. Importantly, control (NK-competent) mice treated with Ad-CMV-p53 (SCH58500) showed no tumor growth or ascites production, and all animals survived the experimental period (240 days). These results indicate that immunological mechanisms involving NK cells play an important role in the bystander effect involving adenovirus-p53 gene therapy for ovarian cancer.

Discussion
Gene therapy is a promising new approach for the treatment of cancer. In animal models, complete elimination of cancer cells is often achieved, although the therapeutic gene has not been delivered to all of the cells (41). This is referred to as a bystander effect because tumor cells near those that receive the therapeutic gene are also eliminated. In humans, accessibility of all cancer cells to a therapeutic gene presents an even greater limitation to gene therapy. Therefore, it is important to understand the basis for bystander effects, to design gene therapy protocols that maximize it, and to develop combination therapies designed to augment or complement mechanisms that are operative in bystander effects.

Several mechanisms have been proposed for the bystander effect, including intercellular communication within the tumor via gap junctions (41), apoptosis (42), antiangiogenesis (26, 38), cytokines or other soluble mediators (43, 44), and immunological mechanisms (45–48). It seems likely that more than one mechanism is operative in most experimental systems (41, 42, 48). Thus in this study, our focus on immune system activation was not based on the assumption that it is the sole mechanism for bystander effects. However, activation of the immune system is appealing as a mechanism to investigate because of the many ways to manipulate the immune system with depleting antibodies or using immunoincompetent mice. In addition, there are a variety of ways in which many immune mechanisms can be maximized or augmented to optimize any bystander effects detected (47).

A number of immunological mechanisms have been implicated in bystander effects in various experimental systems using p53 or HSV-tk as the therapeutic gene (43–47). NK cells have been implicated in the bystander effects noted in
mice treated with adenovirus p53 (47), adenovirus HSV-tk (46), and tumor cells transfected with adenosine deaminase (43). These studies used immunodeficient mice challenged with human tumors (47) or immunocompetent mice challenged with a syngeneic tumor (46). In either case, depletion of NK cells by administration of a monoclonal antibody in vivo (46, 47) or by the use of an NK-deficient mouse strain (47) demonstrated a key role for NK cells in the bystander effect. The results of this study have extended these findings to clearly demonstrate that the efficacy of enhanced survival in vivo using Ad-CMV-p53 (SCH58500), is dependent upon the NK cell population.

NK cells were first characterized to be large granular lymphocytes that secrete cytokines and mediate direct cytotoxicity (49). NK cells can attack normal (nonself) cells under certain conditions, but they generally exhibit stronger activity against tumor cells and virus-infected cells. A key principle of the specificity of NK cells was revealed by studies showing that NK cells prefer to attack cells that have down-regulated classical MHC class I molecules. The ability to spontaneously lyse tumor cells is the best-known functional attribute of NK cells, and particular tumor escape variants that have lost MHC class I expression are efficiently controlled in vivo by NK cells (50). NK cells mainly circulate in the blood, where they account for 5–15% of circulating lymphocytes; however, in certain pathological conditions, including viral and bacterial infections, NK cells selectively accumulate in the infected tissue sites (51). Several studies have shown that NK cells express many known adhesion molecules that bind to ligands present on both resting and inflamed endothelium (52). Furthermore, NK cells express many chemokine receptors and are responsive to several chemokines, including macrophage inflammatory protein-1α, IFN-inducible protein-10, and monocyte chemotactic protein family members (53). Several cytokines have been shown to modulate these adhesive as well as chemotactic functions, including IL-2, IL-4, IL-8, IFN-g, IL-12, and tumor necrosis factor (54, 55). The production and interaction of these cytokines involved in NK cell activity are complex, and the effects of treatment with rAd-null or Ad-CMV-p53 (SCH58500) in the peritoneal cavities of mice are under current investigation. In addition, it will be necessary to characterize the mechanisms leading to NK cell recruitment and activation by adenoviruses.

Increased production of cytokines has been implicated as a mechanism for bystander effects (44), and the antitumor effects of some cytokines have been exploited further by
using viral vectors to deliver cytokine genes to tumor cells in vivo (56, 57). Successful gene therapy outcomes in mice have also been reported using viral vectors to deliver cytokine genes to autologous immune effector cells (58, 59) or to tumor cells ex vivo with subsequent administration to tumor-bearing mice (44). Expression of cytokines such as IL-12 or IL-2 by tumor cells can lead to activation of NK cells (60), and IFN-γ apparently can exert antiangiogenic effects in tumors (61). Delivery of the IL-2 gene to activated NK cells has been used to produce NK cells that retain lytic function in the absence of additional IL-2 (59), and a similar approach has been used to generate activated human NK T cells that produced one complete regression of cancer in a small clinical trial (58). These results are particularly important in view of the observation that i.p. administration of LAK cells plus exogenous IL-2 produced documented responses in ovarian cancer patients (62, 63), but IL-2–related toxicity could not be tolerated by some patients and limited the number of cycles of treatment in other patients. Because most ovarian cell lines and freshly explanted tumor cells from humans are susceptible to the lytic action of LAK cells (64), the use of IL-2–transfected LAK cells to obviate the need for toxic doses of exogenous IL-2 seems particularly promising. A cytokine cascade, macrophage activation, and the migration of macrophages and T lymphocytes into tumors have been implicated in antitumor activity in mice challenged with HSV-tk-modified tumor cells (44, 45, 65). The mechanism of tumor killing in vivo by NK cells may also involve secretion of these cytokines that could act on other immune populations and/or directly on neoplastic cells. It will be important to investigate this mechanism further to improve the therapeutic effect.

The athymic nude mouse is an important and well-characterized test system for novel therapeutic regimens of tumors in vivo, because it allows the testing of human tumor cell lines in a nontoxicogenic host. The results of our study, however, suggest that a syngeneic mouse model system will be important to develop to extend our studies of the bystander effect. However, although mouse ovarian cancer cell lines (either spontaneous or induced) are rare, we are currently working to characterize the response of promising new ovarian cancer cell line to Ad-CMV-p53 (SCH58500).

It is clear that adenovirus vectors commonly used in gene therapy can induce inflammatory responses. Adenovirus vector–induced inflammation has generally been considered an adverse effect in gene therapy, and considerable effort has been expended to develop methods to minimize it (66). However, in the context of gene therapy for ovarian cancer, macrophage recruitment and activation could also be beneficial. One of the adverse effects of adenovirus–based gene therapies is inflammation with an apparent potential for a lethal shock syndrome in rare cases. However, an overlooked advantage of the well known proinflammatory effects of adenoviruses is that they may be used enhance activation of certain antitumor effector cells. One of the hallmarks of inflammation is an early increase in neutrophil infiltration. These studies have focused not on early inflammation, but on later immune responses, namely NK cell recruitment and activation. It will be necessary to include a more complete characterization of the early inflammatory response to adenoviruses in future clinical studies.
A variety of methods have been used to deliver therapeutic genes to tumor cells in a manner that allows gene expression and elimination of tumor cells. The use of the replication-deficient adenovirus vector was one of the first approaches used, and it is one of the most well-developed and studied technologies, with several clinical trials in progress in humans. The i.p. administration of adenovirus vectors for the treatment of cancers in the peritoneal cavity demonstrates a number of potential advantages over other modes of gene therapy. For example, a recent study suggests that i.p. administration of an adenovirus vector induces a substantial systemic antibody response to adenovirus, but antibodies could not be detected in the peritoneal cavity (67). In addition, some immune effector cells are resident in the peritoneal cavity, and these can be activated, and more can be attracted by a variety of stimuli (68). In this context, it is interesting to note that rAd-null alone (a replication-defective adenovirus with no CMV promoter and no transgene expression cassette) is capable of recruiting and activating peritoneal NK cells. However, we cannot rule out, at this point, that intrinsic differences in adenoviruses could affect NK cell recruitment and, hence, efficacy.

It may be possible to enhance further the bystander effect with higher adenovirus doses to increase the survival of animals treated with rAd-null alone. In these studies, we injected animals with adenovirus doses at a ratio of 100 C.I.U./NIH-OVCaR-3 cell injected, a concentration sufficient for 100% infectivity in vitro (15, 16). Although we have attempted a quantitation of infectivity in vivo using marker genes such as β-galactosidase, it is very difficult to ascertain the level of tumor cell infectivity in vivo after injection of adenovirus, because the tumor cells are injected i.p., where they are widely distributed and cannot be quantitated. Our current efforts to monitor infectivity involve labeling tumor cells with a fluorescent dye before injection.

Our results suggest that the bystander effect involving NK cells may play an important role in obtaining a therapeutic response to tumor cells in vivo. It is possible that this effect may also be important in killing tumor cells that are resistant to the direct cytotoxic effect of a particular transgene. Thus the immune response in general and NK cell activation and recruitment in particular, will need additional evaluation in cancer patients who do (or do not) respond to adenovirus-based gene therapy. In addition, there may be ovarian cancer (target) cells that are resistant to NK cell lysis. It is interesting to speculate that such a population may arise in recurrent tumors after the clinical application of adenovirus gene therapy.

The effectiveness of p53 as a therapeutic gene in a number of human ovarian cancer lines has now been clearly demonstrated in vivo (15, 16, 69, 70). Our results demonstrate that immunological mechanisms involving natural killer (NK) cells contribute to the bystander effect in a mouse model for adenovirus p53 gene therapy of ovarian cancer. It is possible that this bystander effect can be exploited to improve the efficacy of therapy.

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