A Fiber-Modified, Secretory Leukoprotease Inhibitor Promoter-Based Conditionally Replicating Adenovirus for Treatment of Ovarian Cancer

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ABSTRACT

Purpose: The use of conditionally replicating adenoviruses (CRAD) is dependent on molecular differences between tumor cells and nontumor cells. Transcriptional targeting of CRAD replication via tumor-specific promoters is an effective way to control replication regulation. Genetic fiber pseudotyping is an approach for circumventing low expression of the primary adenovirus serotype 5 (Ad5) receptor by using the distinct adenovirus serotype 3 (Ad3) receptor for entry into and subsequent killing of ovarian cancer cells.

Experimental Design: In this study, we constructed a fiber-modified CRAD containing the secretory leukoprotease inhibitor (SLPI) promoter to control viral replication via the E1A gene (Ad5/3SLPI). To evaluate the liver toxicity of chimeric 5/3 fiber-modified CRADs, we compared Ad5/3SLPI with Ad5/3Cox-2L, a CRAD with E1A under control of the Cox-2 promoter, and Ad5/3Δ24, a CRAD that replicates in cancer cells inactive in the retinoblastoma/p16 pathway by use of an in vivo hepatotoxicity model and by a model system that uses slices of human liver.

Results: We show efficient viral replication and oncolysis of Ad5/3SLPI in both multiple ovarian cancer cell lines and primary tumor cell spheroids as well as therapeutic efficacy in an orthotopic mouse model of peritoneal carcinomatosis. Ad5/3SLPI showed significantly decreased liver toxicity compared with other 5/3 fiber-modified control vectors examined.

Conclusions: In summary, Ad5/3SLPI is a promising vector candidate for treating metastatic ovarian cancer and showed robust virus replication, oncolysis, and in vivo therapeutic efficacy. Ad5/3SLPI showed comparatively low liver toxicity and therefore holds potential for patient use in the clinic.

INTRODUCTION

Ovarian cancer is the most fatal gynecologic malignancy in the United States, and the incidence is reported to be increasing (1). The majority of patients present with peritoneally disseminated disease, which is associated with a poor prognosis. In this regard, anticancer molecular therapies have been proposed as a treatment alternative for advanced cancers refractory to conventional treatment modalities. To this end, adenoviral vectors have been used for a variety of gene therapy applications (2). Unfortunately, the efficacy of gene transfer to solid tumors has been limited; thus, there is little evidence supporting significant clinical benefit, albeit only phase I and II trials have been reported (3, 4). To overcome this obstacle, conditionally replicating adenoviruses (CRAD) have emerged as novel therapeutic agents for a variety of neoplastic diseases, which led to their rapid translation into clinical trials (5, 6). CRADs have been designed to replicate in tumor cells whereby the viral replication results in oncolysis and subsequent release of the virus progeny (7, 8).

Two strategies have been employed to restrict virus replication to target cells and to spare normal tissue. “Genetic complementation”—type (a.k.a. “type 1”) CRADs (9, 10), such as Ad5Δ24, have a mutation in the immediately early (E1A) or early (E1B) adenoviral region, which is complemented in tumor cells but not in normal cells. In “transcomplementation”—type (a.k.a. “type 2”) CRADs, virus replication is controlled via a tumor/tissue-specific promoter (11). However, in both instances, ectopic liver transduction is the major predicator of adenoviral vector-induced toxicity. Therefore, it is rational to choose a tumor-specific promoter, which is highly expressed in the tumor but has potentially low activity in the liver.

One of the most promising promoters for ovarian cancer is the promoter for secretory leukoprotease inhibitor (SLPI). SLPI

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SLPI Promoter-Based Gene Therapy for Ovarian Cancer

is a 11.7-kDa serine protease inhibitor that has been described to be highly expressed in different human carcinomas, including those derived from breast, lung, endometrium, and ovary (12). SLPI gene expression has been investigated in cell lines and tissues. Northern blot analysis has been employed to measure the levels of SLPI gene expression in SKOV3.ip1 ovarian cancer cells (13). Gene expression analysis has been used to show that SLPI is 60-fold up-regulated in ovarian carcinomas compared with normal ovarian epithelium (14). In addition, SLPI is minimally expressed in the normal liver (15). Finally, quantitative real-time PCR was used to analyze a panel of 39 microdissected ovarian carcinomas for the presence of genes expected to be up-regulated. The SLPI gene was found to be overexpressed at high levels in all of the ovarian cancer subtypes examined (16). Based on these considerations, the SLPI promoter has been investigated recently as a clinically applicable agent and described as useful in the context of ovarian cancer gene therapy (17, 18). Recent studies have shown that the oncolytic potency of a CRAD agent is directly determined by its ability to infect target cells (19). Unfortunately, in the field of ovarian cancer, transduction efficacy by adenovirus serotype 5 (Ad5) is frequently suboptimal due to the highly variable and often low expression pattern of the primary adenovirus receptor, coxsackie adenovirus receptor (CAR; refs. 20–22). In contrast, CAR is expressed ubiquitously on most normal tissues. High liver transduction by adeno viral vectors following systemic administration can lead to severe side effects and therefore restricts i.v. application of adeno viral vectors. Several strategies have been developed to alter the tropism of adenovirus to improve its utility as a gene transfer vector. Increased transduction of low CAR tumor cells can be achieved via genetic reargeting approaches. One such approach is the substitution of Ad5 with a fiber knob deriving from adenovirus serotype 3 (Ad3). Ad5/3 chimeras have displayed enhanced infectivity of ovarian cancer cells without increasing gene delivery to murine livers (23, 24). It was the purpose of this study to develop a novel 5/3 fiber-modified, SLPI-based CRAD (Ad5/3SLPI), and oncolytic activity and selectivity was shown using established ovarian cancer cell lines as well as purified ovarian cancer cells from patients. Potent antitumor efficacy was shown in a xenograft model of i.p. ovarian cancer after i.p. virus injection because the compartmentalized nature of ovarian cancer creates a rationale for locoregional treatment.

MATERIALS AND METHODS

Cell Lines. Hey, OV-4, and SKOV3.ip1 ovarian adenocarcinoma cell lines were kind gifts from Dr. Timothy J. Eberlein (Harvard Medical School, Boston, MA) and Drs. Judy Wolf and Janet Price (both from University of Texas M.D. Anderson Cancer Center, Houston, TX), respectively. The ovarian adenocarcinoma cell line OV-3 was obtained from American Type Culture Collection (Manassas, VA). The lung adenocarcinoma cell line A549 was a kind gift from Dr. A.J. van der Eb (University of Leiden, Leiden, the Netherlands). Cell lines were maintained under recommended conditions. Cells were grown at 37°C in a humidified atmosphere of 5% CO2. Fresh malignant ascites fluid samples from three patients with pathologically confirmed ovarian adenocarcinoma were obtained from the University of Alabama at Birmingham Hospital (Birmingham, AL) following informed consent and institutional review board approval. Cancer cells were purified using a previously described immunomagnetic-based method (24, 25). Briefly, ovarian cancer cells endogenously displaying B72.3 on their cell surfaces were initially bound with a mouse anti–tumor-associated glycoprotein 72 antibody and subsequently collected with magnetic beads coated with anti-mouse IgG. To create three-dimensional spheroids, cells were suspended in growth medium in 3% agar-coated flasks and incubated overnight at 37°C in a 5% CO2 environment on a rocker.

Recombinant Adenoviruses. A fiber shuttle vector, pN E.B.P.K.F5/3, containing an Ad5 tail and shaft and an Ad3 knob was digested with EagI and KpnI and used for homologous recombination with SvaI linearized plasmid pVK500, generating pAdback5/3. Subsequent homologous recombination with shuttle plasmids (26) pS ESLPI or pSECo x-2L that contained the E1A gene downstream of the 1,320-bp SLPI promoter (17) or Cox-2L promoter (11) resulted in plasmids pAd5/3SLPI or pAd5/3Cox-2L, respectively, which contained the recombinant adenoviral genomes. Plasmids were validated for promoter insertion and fiber gene modification by PCR and restriction digest. Adenoviral particles were produced by transfection of FacI-digested pAd plasmids into HeLa cells using LipofectAMINE (Life Technologies, Rockville, MD) following the manufacturer’s protocol. The presence of the E3 region and the 5/3 fiber knob modification were confirmed with PCR. The absence of wild-type E1A was confirmed with PCR. Generation of recombinant adenoviruses Ad5/3wt, Ad5/3Δ24, and Ad5/3Luc was described elsewhere (23, 27, 28). All vectors contain chimeric fibers with the tail and shaft domains of Ad5 and the knob domain of Ad3. Ad5/3wt contains the wild-type Ad5 genome, and Ad5/3Δ24 contains a 24-bp deletion in the constant region 2 of E1A. Ad5/3Luc is a replication-incompetent adenoviral vector containing a firefly luciferase transgene cassette in place of the deleted E1 region. All replicative adenoviruses were amplified on HeLa cells to avoid wild-type contamination. Replication-deficient, E1-deleted Ad5/3Luc has been amplified in 293 cells. Purification was done with double CsCl gradients using standard methods. The viral particle (vp) concentration was determined at 260 nm, and standard plaque assay on 291 cells was done to determine infectious particles. The ratios of vp to infectious particles were 9.8, 12.4, 7.6, 4.7, and 45.7 for Ad5/3SLPI, Ad5/3Cox-2L, Ad5/3wt, Ad5/3Δ24, and Ad5/3Luc, respectively.

In vitro Cytotoxicity Assay. Cells in triplicate were infected for 1 hour at 37°C in 50 μL growth medium with 2% fetal bovine serum. Thereafter, cells were incubated with 5% growth medium. Cell viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay, Promega, Madison, WI] on day 6 (SKOV3.ip1 and OV-3), day 10 (Hey), or day 13 (OV-4) as described previously (27). The results with Ad5/3SLPI were compared with those of the other viruses using two-tailed Student’s t test. For crystal violet staining (29), cells were plated and infected on six-well plates, and infection volume was brought to 500 μL. Oncolysis was evaluated when Ad5wt or Ad5/3wt showed clear oncolysis with the lowest amount of virus.
Quantitating Virus Replication. Primary ovarian cancer cells were purified and cultured as spheroids overnight. The next day, spheroids were infected with 1,000 vp/cell with Ad5wt, Ad5/3wt, Ad5/3SLPI, Ad5/3luc, or no virus. Then, the spheroids were divided into aliquots of 10^5 cells in Costar 96-well ultralow attachment plates (Corning, Inc., Corning, NY). Cells and growth medium were harvested together and frozen at 1, 2, 3, 4, and 5 days after infection. Purification of the DNA and quantitative PCR for E4 were done as described previously (27). To quantitate the total E4 copy number, DNA was purified from the spheroid suspension (cellular and growth medium fractions) using a DNasey Tissue kit (Qiagen). The primers used for amplifying the E4 were forward 5'-GGAGTGGCGGAGACAACTC-3' and reverse 5'-ACTACGTCGCGGTTCCATT-3' and detected with the probe 5'-TGGCATGACACTACGACCAAC-3'. Human β-actin was amplified to control for housekeeping gene copies within the cells as described previously (30).

Therapeutic Ovarian Cancer Model. Mice were obtained at 3 to 4 weeks of age and quarantined at least 1 week before beginning the study. Mice were kept under pathogen-free conditions according to the American Association for Accreditation of Laboratory Animal Care guidelines. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of University of Alabama at Birmingham. Female CB17 severe combined immunodeficient mice (University of Alabama at Birmingham Centers for AIDS Research Mouse Core Facility) were injected i.p. with 1 × 10^7 SKOV3.ip1 cells on day 0. On days 10, 11, and 12, mice were injected i.p. with 1 × 10^5 vp of Ad5/3SLPI (n = 10), Ad5/3Cox-2 (n = 10), Ad5/3wt (n = 10), Ad5wt (n = 10), Ad5/3luc (n = 9), or no virus (n = 9) in 500 µL Opti-MEM (Mediatech, Herndon, VA). In another experiment, mice were injected i.p. on days 10 and 11 with 5 × 10^5 vp of Ad5/3SLPI (n = 10), Ad5/3Cox-2 (n = 10), Ad5/3wt (n = 10), Ad5wt (n = 10), Ad5/3luc (n = 9), or no virus (n = 9) in 500 µL Opti-MEM. Mice were followed daily and killed when there was any evidence of pain or distress. Survival data were plotted as a Kaplan-Meier curve, and the Ad5/3SLPI group was compared with the other groups using log-rank analysis and χ² testing via Prism 4 software (GraphPad Software, San Diego, CA).

In vivo Toxicity Study. To evaluate the potential liver toxicity of different 5/3 fiber-modified CRADs in vivo, female C57BL/6 mice were infected i.p. with 5 × 10^10 vp of Ad5/3SLPI, Ad5/3Cox-2, Ad5/3-D24, Ad5/3wt, Ad5wt, and Ad5/3luc. After 48 hours, mice were killed and the livers were harvested and fixed in 10% buffered formalin. Serial paraffin-embedded sections were taken and stained with H&E under standard conditions. Histopathology was scored in a blinded manner by an expert pathologist (G.P.S.). Two other liver samples were snap frozen on dry ice. To analyze the E1A RNA level, total RNA from the liver was extracted with a RNeasy Mini kit (Qiagen). A GeneAmp RNA PCR core kit (Applied Biosystems, Foster City, CA) was used for cDNA synthesis and PCR amplification of cDNA products. Taqman primers and probes were designed by the Primer Express 1.0 software and synthesized by Applied Biosystems. Oligonucleotide sequences for amplification of the E1A gene were forward primer AACCAGTTGCGTGAGAGTTG, reverse primer CTCGTATAGCAGTCCTCGATA, and probe 6FAM-CACAGCCTGGCGACGCTA-TAMRA. The human housekeeping gene GAPDH was used as an internal control. The sequences to amplify GAPDH gene were forward primer GGTTTACATGTACCATTGATCCCA, reverse primer ATGGGATTTTCATTGATACCAAG, and probe 6FAM-CGTTTCTCGGTGAGCTGACTCAT-TAMRA. With optimized concentration of primers and probes, the components of the real-time PCR mixture were designed to result in a master mix with a final volume of 9 µL per reaction containing 1 × Taqman EZ RT-PCR kit (Applied Biosystems), 100 nmol/L forward primer, 100 nmol/L reverse primer, 100 nmol/L probe, and 0.025% bovine serum albumin. Total RNA sample (1 µL) was added to 9 µL PCR mixture in each reaction capillary. A negative control (no template) received 1 µL water. For the assay, a known amount of E1A RNA (10^5, 10^6, 10^7, and 10^8 copies per µL) was amplified to generate a standard curve for quantification of the E1A copy numbers. Capillaries were sealed and centrifuged using a LC Carousel Centrifuge (Roche Molecular Biochemicals, Indianapolis, IN) to facilitate mixing. All PCR reactions were carried out using a LightCycler System (Roche Molecular Biochemicals). Thermal cycling conditions were 2 minutes at 50°C, 30 minutes at 60°C, 5 minutes at 95°C, and 40 cycles of 20 seconds at 94°C and 1 minute at 60°C. Data were analyzed with LightCycler software.

Precision-Cut Human Liver Slices. Human liver tissue was obtained from livers taken from multiorgan donors following institutional review board approval. Informed consent was obtained from the legal next-of-kin for the explantation of organs for transplantation purposes. Excess material was received from donor livers reduced in size to perform transplantation at the University of Alabama at Birmingham Hospital. The donor livers were perfused in situ with University of Wisconsin solution at 4°C and kept on ice until reduction. Precision-cut liver slices (diameter 4 mm, thickness 150 µm) were prepared using a Krumdiek Tissue Slicer (Alabama Research and Development, Munford, AL). Each slice was transferred into a well of a 24-well plate containing 1 mL William’s Medium E and placed on a rocker. Liver slices were maintained at 37°C in a 5% CO₂ environment on a rocker and allowed to preincubate for 2 hours before treatment. Liver slices were infected with 50 vp/cell with Ad5wt, Ad5/3wt, Ad5/3Cox-2, Ad5/3SLPI, Ad5/3Δ24, Ad5/3luc, or no virus. Liver slices were harvested and frozen at 12, 24, 36, and 48 hours after infection. To analyze the E1A RNA level, total RNA was extracted from the liver using a RNeasy Mini kit (Qiagen). Quantitative reverse transcription-PCR was done as described above.

RESULTS

In vitro Oncolytic Potency of Ad5/3SLPI. To analyze the specificity of Ad5/3SLPI, we infected monolayers of HeLa cells, which have been reported to express high levels of the SLPI gene, and A549 cells, which express low levels of the SLPI gene, as positive and negative control cell line (17), respectively. In both cell lines, the crystal violet staining–based cell killing assay showed complete oncolysis with Ad5/3wt and Ad5wt...
SLPI Promoter-Based Gene Therapy for Ovarian Cancer

Ad5/3SLPI displayed efficient killing of ovarian cancer cells in vitro. We infected monolayers of SKOV3.ip1, OV-4, OV-3, and Hey cells (Fig. 2) with Ad5/3SLPI, Ad5/3wt, or Ad5/3luc. In all cell lines, the quantitative cell killing assay showed no effect with the nonreplicative control. The percentages of viable cells remaining with Ad5/3SLPI were 0%, 5.2%, 8.2%, and 12% for SKOV3.ip1, OV-4, OV-3, and Hey cells, respectively. The percentages of viable cells remaining with Ad5/3wt were 5%, 4%, 42%, and 2% compared with uninfected cells, with SKOV3.ip1, OV-4, OV-3, and Hey cells, respectively. Further, Ad5/3SLPI killed significantly more SKOV3.ip1, OV-4, and OV-3 cells (P < 0.001) than Ad5/3luc. In all ovarian cancer cell lines, Ad5/3SLPI displayed enhanced cell killing compared with Ad5wt (P < 0.001, P < 0.002, P < 0.001, and P < 0.001 for SKOV3.ip1, OV-4, and OV-3 cells, respectively). These results thus showed efficient cell killing efficacy of Ad5/3SLPI in ovarian cancer cells in vitro.

Ad5/3SLPI replicates in ovarian cancer primary cell spheroids. We analyzed three purified, unpassaged human primary ovarian cancer samples for adenovirus replication. To measure viral copy number, we collected spheroids and growth medium at indicated time points and did quantitative PCR for the adenoviral E4 gene (Fig. 3). To determine the relative increase in copies, we normalized E4 copy number at each time point to the copy number obtained with Ad5/3luc at 24 hours. On day 5, Ad5/3SLPI copy number had increased to 1,002-, 9,682-, and 9,549-, 31,335-, and 49,480-fold in patient samples 1 to 3, respectively, compared with Ad5/3luc copy number obtained with Ad5/3luc at 24 hours. On day 5, Ad5/3SLPI displayed significantly higher E1A RNA levels than Ad5/3wt, Ad5/3Cox2L, and Ad5/3luc (P = 0.786). A higher dose divided into two injections on 2 consecutive days was also tested (Fig. 4B). For Ad5/3SLPI, Ad5/3Cox2L, Ad5/3wt, Ad5/3luc, and no virus, the median survival of mice was 91, 89, 102, 67, 32, and 30 days, respectively. Pairwise χ² testing confirmed significantly improved survival with Ad5/3SLPI compared with Ad5/3luc and no virus (P ≤ 0.0001) and Ad5/3wt (P = 0.0188). Interestingly, survival of mice treated with Ad5/3wt in the low-dose group was significantly reduced compared with the high-dose group (P = 0.0181). In contrast, the survival of mice treated with high or low doses of Ad5/3SLPI, Ad5/3Cox2L, or Ad5/3wt did not differ significantly.

Evaluation of Liver Toxicity In vivo. To evaluate the liver toxicity of human CRADs preclinically is a challenge. It is known that human adenoviruses do not replicate productively in mice. However, to obtain key information regarding replication specificity of different 5/3 fiber-modified CRADs, we used an in vivo system that relies on histopathologic analysis and quantification of E1A mRNA after systemic administration of viral vectors in immunocompetent C57BL/6 mice (31). We injected 5 × 10ⁱ⁰ vp/mouse of Ad5/3SLPI, Ad5/3Cox2L, and Ad5/3Δ24 or control vectors (Ad5wt, Ad5/3wt, and Ad5/3luc) i.v., and the mice were killed 3 days later for analysis. The liver tissue was processed for histopathologic analysis with H&E staining and adenosivial E1A mRNA analysis. By histopathologic analysis, systemic administration of Ad5/3SLPI, Ad5wt, and Ad5/3luc did not result in any remarkable liver toxicity (Fig. 5A). Histopathology of the livers from mice, which were treated with Ad5/3Cox2L, displayed chronic inflammation within the parenchyma and cell dropout, and Ad5/3Δ24-treated mice displayed similar diffuse chronic inflammation and signs of apoptosis. The highest liver toxicity was seen in mice treated with Ad5/3wt, showing submassive hepatic necrosis and associated congestion and hemorrhage.

Next, we correlated the results of the histopathologic analysis with quantitative E1A mRNA expression in the liver. Mice treated with Ad5/3SLPI, Ad5wt, and Ad5/3luc displayed the lowest levels of E1A mRNA with 19,574 ± 9,549, 31,335 ± 3,130, and 49,480 ± 16,063 mRNA copies, respectively. In contrast, E1A mRNA levels in Ad5/3wt, Ad5/3Cox2L, and Ad5/3Δ24 treatment groups on day 3 (Fig. 5B) were significantly higher with 30,690,000 ± 451,141, 4,557,244 ± 621,139, and 4,330,400 ± 16,063 copies, respectively. Of note, the E1A RNA levels of Ad5/3SLPI-treated animals, where the E1 gene is under control of the SLPI promoter, displayed significantly lower E1A RNA levels than Ad5/3Cox2L and Ad5/3Δ24 (P < 0.001). These data support the
concept that E1A itself, even in the absence of replication, may elicit toxicity and suggest that the SLPI promoter, in the context of a 5/3 fiber-modified CRAD, was minimally active in the mouse liver at the doses evaluated.

Evaluation of Liver Toxicity in Human Liver Slices. To evaluate liver toxicity in the most relevant context, the human liver, we analyzed fresh-cut human liver slices for adenovirus replication employing a novel technique. To measure E1A mRNA, liver slices were collected at 12, 24, and 36 hours (sample 1) and 12, 24, 36, and 48 hours (sample 2) after infection and quantitative reverse transcription-PCR was done to quantitate E1A mRNA (Fig. 6). Ad5/3SLPI, Ad5/3Cox-2L, and Ad5wt displayed the lowest mRNA copy numbers with 2,062, 3,840, and 3,041 copies after 36 hours in sample 1 and 6,709, 103,717, and 22,309 copies after 48 hours in sample 2. Ad5/3wt and Ad5/3D24 displayed significantly higher E1A mRNA copy numbers with 9,061 and 253,550 copies for Ad5/3wt in samples 1 and 2, respectively, and 5,274 and 161,412 copies for Ad5/3D24 in samples 1 and 2, respectively. Compared with Ad5/3SLPI, E1A mRNA copy numbers for Ad5/3Cox-2L, Ad5/3wt, Ad5/3D24, and Ad5wt were increased 1.8-, 4.4-, 2.6-, and 1.5-fold after 36 hours in sample 1 and 15.5-, 37.8-, 24.1-, and 3.3-fold after 48 hours in sample 2, respectively. Importantly, there was good correlation between data obtained from human and mouse livers (Fig. 5).

DISCUSSION

Gene therapy applications in which viral vectors are used for gene transfer have shown promise in preclinical studies. Unfortunately, inefficient tumor transduction has often precluded significant benefit in clinical trials. CRADs have been developed to achieve a higher therapeutic index than for the currently available modalities. However, only phase I and II trials have been done thus far. Importantly, the safety data have been good, but the preliminary evidence of efficacy has not been dramatic. Nevertheless, the emergence of infectivity was enhanced and more oncolytic CRADs may provide enhanced efficacy, which might in turn require more specificity.

In this study, we evaluated the efficacy and efficacy of a CRAD using the SLPI promoter for controlling E1A expression. SLPI was identified as a potent inhibitor of leukocyte serine proteases (32). Although human SLPI has been intensively studied, its precise function in vivo remains unclear. However, SLPI is expressed in ovarian cancer and has low expression levels in normal organs, such as the liver (13, 14, 16). The SLPI promoter retains its fidelity in adenoviral vectors and is activated in ovarian cancer cell lines and ovarian cancer primary cells (17). Furthermore, its activity is low in the liver, which is important concerning safety issues for further clinical evaluations.

The efficacy of CRADs is determined by their infectivity. One method for circumventing the frequent deficiency of CAR on clinical ovarian cancers is genetic retargeting via substitution of the knob domain of Ad5 with a knob from an alternative adenovirus serotype. Earlier studies have shown the increased expression of Ad3 receptors compared with Ad5 receptors in ovarian adenocarcinoma cell lines (24). 5/3 Fiber-modified adenoviruses (23, 27, 33) have shown improved targeting to ovarian cancer cell lines and freshly isolated ovarian cancer tissue from patients. It is conceivable that the Ad3 receptor, as opposed to CAR, may not be involved in the carcinogenic process and thus not down-regulated in advanced tumors. Here, we did a preclinical evaluation of Ad5/3SLPI in the context of ovarian cancer gene therapy. We evaluated the cell killing efficacy of Ad5/3SLPI in four ovarian cancer adenocarcinoma cell lines, which have been shown to express SLPI or allow SLPI-controlled transgene expression (17, 18). In a quantitative...
assay, Ad5/3SLPI displayed enhanced oncolysis when compared with a wild-type adenovirus, whereas Ad5/3wt achieved almost total oncolysis with all cell lines (Fig. 2). In a crystal violet staining assay, Ad5/3SLPI displayed specific tumor cell killing only in a SLPI positive cell line. The cell killing efficacy of Ad5/3SLPI correlated with the replication rate in three-dimensional human ovarian primary cancer cell spheroids.

Finally, the therapeutic efficacy of Ad5/3SLPI was evaluated in an orthotopic mouse model of ovarian cancer. Xenograft models are a useful tool to study adenoviral transduction of tumor cells and viral replication in tumors. Most current xenograft tumor models are based on s.c. transplantation of established human tumor cell lines into immunodeficient mice and subsequent intratumoral vector injection. In the context of ovarian cancer, these models do not mimic the most relevant clinical situation. Therefore, we have used a model of i.p. cancer and i.p. injection of viral vectors. Ad5/3SLPI displayed significantly enhanced survival in comparison with Ad5wt and the nonreplicative negative control Ad5/3luc. Survival of mice treated with Ad5/3wt and Ad5/3Cox-2L, another promising CRAD with E1A under control of the Cox-2 promoter, was comparable with mice treated with Ad5/3SLPI. In summary, these results indicate a comparable therapeutic efficacy for S/3 fiber-modified adenoviruses driven by the SLPI or the Cox-2L promoter. Converted weight/weight into humans, the lower dose used in our study would equal $9 \times 10^{10}$ vp. This is well below the $2 \times 10^{12}$ vp daily for 5 consecutive days, without dose-limiting toxicity (34). In vector trials, up to $7.5 \times 10^{13}$ vp have

Fig. 3 Ad5/3SLPI replicates in three-dimensional human ovarian primary cancer cell spheroids. Purified and unpussaged ovarian cancer cells were allowed to form spheroids, which were infected with 1,000 vp/cell of Ad5/3SLPI, Ad5/3wt, Ad5wt, and Ad5/3luc (E1-deleted control virus). Spheroids and growth medium were collected at indicated time points, and virus copy number was measured with quantitative PCR. A–C, increase in virus copy number is noted in patient samples 1–3, respectively. Background values (uninfected spheroids) were subtracted. Bars, SD.

Fig. 4 Therapeutic effect of Ad5/3SLPI in an animal model of peritoneally disseminated ovarian cancer. SKOV3.ip1 cells were injected i.p. into severe combined immunodeficient mice, and advanced carcinomatosis was allowed to develop for 10 days. A, mice received three daily i.p. injections of $1 \times 10^{5}$ vp of Ad5/3SLPI, Ad5/3Cox-2, Ad5/3wt, Ad5wt, Ad5/3luc, or no virus or (B) two daily i.p. injections of $5 \times 10^{5}$ vp. In both experiments, Ad5/3SLPI resulted in significantly enhanced survival (A) versus Ad5/3luc, no virus, and Ad5wt (all $P < 0.0001$) and (B) versus Ad5wt ($P = 0.0008$).
gene therapy vectors could be toxicity resulting from infection of normal cells. Previous studies have shown improved tumor transduction with fiber-modified adenoviruses, but the liver toxicity of 5/3 fiber-modified CRADs has not been evaluated systematically. Therefore, we have compared Ad5/3SLPI and Ad5/3Cox-2L, two transcomplementation-type CRADs with E1A under control of the SLPI and Cox-2L promoters, respectively. Another control virus was Ad5/3Δ24, a oncolytic genetic complementation–type CRAD that contains a 24-bp deletion in the constant region 2 of E1A. The expressed protein is unable to bind retinoblastoma protein for induction of S phase (10). Thus, Ad5/3Δ24 is attenuated in nondividing normal cells but replicates in cells inactive in the retinoblastoma/p16 pathway (40). It has been suggested that all human cancers, including ovarian cancers, may be deficient in this crucial pathway (41). Because human adenoviruses do not replicate productively in rodents or other laboratory animals (42), there is no practical model to directly analyze adenoviral replication or adenoviral replication–based toxicities. However, it is well known that adenoviruses efficiently transduce the mouse liver after i.v. administration and produce clinical signs of hepatotoxicity within a week (43, 44). Some of the hepatotoxic effects can be attributed to the expression of the adenoviral E1A gene. For this reason, we have used an in vivo assay of E1A-related hepatotoxicity to evaluate the activity of different promoter-controlled, 5/3 fiber-modified CRADs in normal liver cells in immunocompetent C57BL/6 mice. In this study, we used molecular and histopathologic variables of hepatotoxicity as

been given for 5 consecutive days, without dose-limiting toxicity (35). This equals approximately the dose that we used in the murine toxicity model of our study.

During preparation of this article, another report (36) was published describing a adenovector with a double expression cassette consisting of E1A driven by the SLPI promoter that was and followed by E1B-19K under control of the cytomegalovirus promoter. This vector showed efficacy and specificity in non–small cell lung cancer cell lines in vitro and in a s.c. model of non–small cell lung cancer. Interestingly, the investigators had also deleted the E1B-55K protein and E3, both of which are important for effective oncolysis (37, 38). Transductional targeting of the virus was not endeavored, and replication was atenuated compared with a wild-type virus, although only an in vitro comparison was done. CRADs have emerged as a tool to overcome low tumor transduction demonstrating preliminary evidence of efficacy in clinical trials. In a phase II study of an intratumorally given E1B−55K-deleted CRAD in 40 patients with head and neck cancer, three complete and two partial responses were reported (39). To improve infectivity of target cells, infectivity enhanced CRADs have been constructed. Promising results with such an infectivity enhanced CRAD (Ad5-Δ24RGD) have been reported recently in the context of ovarian cancer gene therapy (29). Ad5-Δ24RGD features a RGD–4C modification in the HI loop of the knob, which allows binding to αvβ3 integrins, which are often overexpressed on ovarian cancer cells. The virus showed impressive oncolysis and enhanced survival in an animal model. Consequently, a clinical phase I trial using this virus for ovarian cancer is in preparation. However, other studies have reported superior tumor transduction rates of 5/3 fiber-modified CRADs compared with CRADs containing a RGD–4C modification. A major challenge for systemic administration of adenovirus-based

### Fig. 5
Ad5/3SLPI displays low liver toxicity in a panel of 5/3 fiber-modified adenoviruses. Female C57BL/6 mice treated i.v. with $5 \times 10^{10}$ vp of Ad5/3SLPI, Ad5/3Cox-2, Ad5/3Δ24, Ad5/3wt, and Ad5/3luc. After 48 hours, the livers were harvested and fixed in 10% buffered formalin. A, serial paraffin–embedded sections were taken and stained with H&E under standard conditions. Histopathology was scored in a blinded manner by an experienced pathologist. B, RNA was isolated from the liver and analyzed by quantitative reverse-transcription PCR for the E1A gene. The result is indicated as E1A RNA copy number per µg total RNA. Columns, mean of three experiments; bars, SD. *, $P < 0.05$ versus Ad5/3luc.

### Fig. 6
Hepatic expression of E1A mRNA of 5/3 fiber-modified adenoviruses in human liver correlates with results obtained in the in vivo mouse model. Precision-cut liver slices were infected with 50 vp/cell with Ad5wt, Ad5/3wt, Ad5/3Cox-2, Ad5/3SLPI, Ad5/3Δ24, or Ad5/3luc. Liver slices were harvested and frozen at 12, 24, and 36 hours (A) or 12, 24, 36, and 48 hours (B) after infection. RNA was isolated from the liver and analyzed by quantitative reverse-transcription PCR for the E1A gene. Points, mean of three experiments.
end points for the activity of heterologous tumor-selective promoters regulating E1A expression. We found that Ad5/3SLPI expressed significantly less E1A mRNA–related hepatotoxicity than Ad5/3wt, Ad5/3Cox-2L, and Ad5/3Δ24.

Of note, all 5/3 chimeric adenoviruses, with the exception of Ad5/3SLPI, seemed to display more liver toxicity compared with Ad5wt. Previous studies (23, 33, 45) have shown an increased liver tropism of 5/3 fiber-modified viruses compared with Ad5 using reporter gene analysis. Interestingly, Kanerva et al. compared viral genomes and subsequent transgene expression and found discrepancy. It was speculated that 5/3 viruses may transduce hepatocytes more readily than Ad5, which might be mostly cleared by Kupffer cells (23). As Kupffer cell uptake would not allow effective viral gene expression, this might partly explain why more E1A mRNA copies were seen after infection with Ad5/3wt in comparison with Ad5wt. However, as Kupffer cells have a central role in determining toxicity (46), it is not clear which is less transducible, transduction of Kupffer cells or hepatocytes. Further, murine and human livers also contain other cell types, such as fibroblasts and endothelial cells. Ad5/3-modified viruses might have improved access to these cells, which might contribute to E1A mRNA expression. Finally, high E1A mRNA levels found after infection with Ad5/3Δ24 are of concern. However, if there is a negative feedback loop associated with the cross-regulation of adenovirus early genes, it may be conceivable that accumulation of E1A mRNA might in fact indicate attenuation of replication. In other words, the lack of productive replication might result in the virus “trying to force the issue” by increased production of E1A mRNA. However, this needs to be studied further.

These results suggest the utility of effective transcriptional control via tumor-specific promoters, such as SLPI. We hypothesize that the comparatively low hepatotoxicity of the Ad5/3SLPI-treated animals was not the result of a failure of the human SLPI promoter to function in murine cells, because the human and murine SLPI promoters share a high degree of similarity (47). To further evaluate differences in the hepatic activity of tumor-specific promoters in the context of fiber-modified adenoviruses, we chose precision-cut human liver slices as a stringent preclinical model. Liver slices have been used previously to evaluate liver function, toxicity, and metabolism (43) and they form a bridge for comparison between animals and humans in toxicology and metabolism studies (44). Precision-cut liver slices maintain the tissue architecture and contain the variety of cell types normally found in the liver. We did a time course evaluation of E1A RNA in liver slices following infection with viral vectors. Importantly, the E1A RNA levels of 5/3 fiber-modified adenoviruses correlated with results obtained from mouse livers following i.v. administration. Nevertheless, it is not known how much E1A expression is required for effective replication. Therefore, it cannot be excluded that there was productive replication of Ad5wt although E1A mRNA expression was low.

In conclusion, our results suggest that Ad5/3SLPI allows tumor-specific replication and improved cell killing compared with wild-type adenovirus. We showed decreased liver tropism and toxicity compared with Ad5/3Cox-2L and Ad5/3Δ24. Chimeric 5/3 fiber-modified CRADs are effective agents for treatment of ovarian cancer. To make gene therapy not only feasible but also clinically useful, strategies to ensure both safety and efficacy are needed. In this regard, Ad5/3SLPI is promising as an agent for achieving enhanced but selective replication, which could reduce adverse effects in clinical trials. We believe that Ad5/3SLPI could be an effective and safe gene therapy agent for treatment of ovarian cancer or other tumors featuring high expression of SLPI and low expression of CAR.

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REFERENCES


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