A Novel Assay to Assess Primary Human Cancer Infectibility by Replication-Selective Oncolytic Adenoviruses

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ABSTRACT

Purpose: Replication-selective oncolytic adenoviruses hold promise for cancer treatment, but the predictive use of cell lines, dissociated tumor tissue, and animal models for efficacy against primary cancers are unclear. To further evaluate cytotoxicity and the potential for efficacy of replication-competent adenoviruses we therefore developed a novel methodology using primary human cancer specimens ex vivo; ovarian, colon, rectal, and breast carcinomas were included.

Experimental Design: Tissue culture conditions were developed to maintain viability of adenocarcinomas ex vivo for 48 hours postsurgery. Explants were infected by replication-competent (wild type 5 and E1A mutant dl222-947) and replication-defective (dl312) adenoviruses; early (E1A) and late (hexon) viral gene expression, αv integrins, coxsackievirus and adenovirus receptor (CAR) and tissue viability were assessed by immunohistochemistry and histopathology. Viral replication was verified by replication assays on selected samples.

Results: Viral gene expression varied dramatically among cancer specimens (n = 41). With Ad5, hexon expression was high in 8 of 11 tested specimens, whereas E1A levels were detectable in 16 of 27 tumor explants. Viral gene expression, distribution, and cytopathic effects were greater postinfection with dl222-947. Specimens that supported early gene expression (E1A) also supported viral replication in 13 of 14 tested cases, determined by recovery of infectious units. As predicted, the replication-defective adenovirus dl312 was not associated with viral gene expression.

Conclusions: Primary human tumor tissue remained viable when cultured ex vivo enabling evaluation of viral mutants in tissue with intact morphology. This assay may have great use in determining treatment-sensitive cancers and assess specific oncolytic mutants in individual cases.

INTRODUCTION

Treatment of most cancers currently requires multimodal- ity therapies including surgery, radiotherapy, and chemotherapy regimens. Colorectal and mammary epithelial carcinomas are two of the most frequently occurring cancers in both the United States and Europe (1, 2). The incidence of ovarian cancer is less frequent but is still a leading cause of cancer-related death in women due to problematic and delayed diagnosis. Once diagnosis is made the disease has, in most cases, already spread to the peritoneal cavity with metastasis in other organs (3–6). Most frequently, combination therapies with a battery of cytotoxic drugs are administered to all patients with cancer. For ovarian cancers, platinum-based cytotoxic drugs and taxanes (3, 4) are frequently combined; for colorectal tumors, the choice is often 5-fluorouracil and leucovorin (7); and for breast cancer, combinations of anthracyclins, taxanes, doxorubicin, and cyclophosphamid are administered (8). The initial response rate to chemotherapy is often good, with rates greater than 60% for ovarian cancers. Despite the initial promising response to treatment, drug resistance often develops within months resulting in a 5-year survival rate of less than 28% for ovarian and a 50% death rate from recurrent disease in patients with colorectal carcinoma (6, 9). Recently, additional cytotoxic drugs have been developed. Some examples are the p.o. fluoropyrimidine prodrugs, oxaloplatin, irinotecan (CPT-11), and gemcitabine as well as monoclonal antibody therapies such as trastuzumab (Herceptin), cetuximab (Erbitux), and bevacizu- mab (Avastatin) (7, 8, 10–12). However, treatment with these drugs also eventually results in cross-resistance. Consequently, novel cancer therapies that lack this cross-resistance are needed and are being developed for use in conjunction with standard treatment regimens.

Replication-selective oncolytic viruses, including adenovi- ruses, represent one promising, novel therapeutic platform for cancer that lacks cross-resistance with cytotoxic drugs (13–15). Adenoviral mutants engineered to target tumor tissue for selective replication and amplification at the tumor site with minimal replication in normal tissue can result in efficient clearance and reduced toxicity. The enhancement of safety and antitumoral potency of mutant viruses was made possible through the observation that many of the critical regulatory proteins that are inactivated by viral gene products in normal tissue are already inactivated during carcinogenesis (16–19). Therefore, deletion of viral genes essential for replication can
be complemented in the cancer cells but not in normal cells (20–22). This concept was first exploited for a mutant virus with the E1B55Kd gene deleted (dl1520, or Onyx-015) reported to be selective for cells lacking p53 function, although other cellular genetic factors are also clearly involved (20, 23, 24). Definitive data are now available from numerous phase I/II clinical trials proving the dl1520 mutant virus to be well tolerated by all routes of administration (25–27). Efficacy was improved when this virus was used in conjunction with standard cytotoxic agents such as cisplatin and 5-fluorouracil both in cells in culture and in nude mice xenografts as well as in individual cases in clinical trials (27–29).

Several adenoviral mutants are now being developed for clinical testing by targeting other cellular proteins involved in tumorigenesis, for example, the pRb pathway, or tissue-specific promoter-driven viruses targeting prostate (CN706, CN787), bladder (CG8840), and hepatocellular carcinomas (AvE1a04i) (21, 22, 30–33). One such deletion mutant is the d922-947 virus and its homologue Ad5A24, carrying deletions in the CR2 domain of the E1A gene (21, 22). The CR2 region is essential for binding of the retinoblastoma tumor suppressor family proteins (Rb) for induction of S phase to support viral replication (34). Consequently, the dl922-947 virus replicates poorly in non-cycling normal cells, whereas replication in cycling tumor cells with deregulated Rb pathway can proceed. Differently from the deletion of the entire EIB55K gene in the dl1520 mutant, the small deletion in the dl922-947 virus does not lower replication efficacy. The mutant virus has been shown to readily infect, replicate, and kill tumor cells both in vitro and in vivo at levels equal to or greater than both wild-type virus and the dl1520 mutant (21).

Abnormalities in the p53 and Rb pathways have been reported for most adenocarcinomas including colorectal, ovarian, and mammary epithelial tumors. Mutations in the Rb pathway including the CDK4 (p16 and p15) inhibitor genes have frequently been detected in ovarian carcinoma cell lines both in vitro and ovarian tumors in situ (35–39). Preclinical data from ovarian carcinoma xenografts in mice treated with either the dl1520 mutant or a retargeted E1A-CR2 deleted mutant (Ad5-A24RFD) showed efficacy and enhanced survival compared with untreated animals (29, 40, 41). Other data from colorectal and mammary carcinoma xenografts in nude mice also showed superior efficacy and improved survival when treated with the dl922-947 mutant (21). Although many novel adenoviral mutants have shown promise as future oncolytic agents in preclinical studies, clinical results have thus far been disappointing; no responses were reported from a phase I trial with i.p. administration of Onyx-015 in patients with recurrent ovarian carcinomas (26). A major reason for this difference in efficacy is that cancer cell lines, passed in vitro for years, might not reflect the biology of tumors in vivo, making the applicability of preclinical results to the clinical situation unclear. Another major reason is that human tumors usually show clinical and genetic heterogeneity, and successful treatment in one case might not be efficacious in other patients. It is essential to address these issues in the evaluation of any novel anticancer treatment prior to clinical trials. This highlights the need to carefully screen the optimal target cancers for different oncolytic agents and the inability to do this with currently available preclinical models.

Here we report on the development of an ex vivo culture system to assess adenoviral infection and replication in various human adenocarcinoma explants. We evaluated the infectivity and sensitivity of each tumor specimen to replication-competent wild-type and replication-selective adenoviruses in this system. Tissue survival up to 48 hours was shown and early and late viral gene expression was detectable from 17 to 48 hours in the carcinoma cells of the tissue samples. Coxackievirus and adenovirus receptor (CAR) and αv integrin expression was also evaluated. We suggest this ex vivo method as a relevant experimental model, more similar to the clinical situation than current models. The method could be further developed for the testing of oncolytic adenoviruses in individual cases in the clinic once efficacy and toxicity have been evaluated in vitro and in vivo. The predictive value of the assay can be tested in future clinical trials to determine if results obtained ex vivo can predict for clinical efficacy and whether the assay potentially could become valuable for patient selection.

MATERIALS AND METHODS

Tumor Tissue. Human carcinoma and normal tissues were obtained with the approval of Local Research Ethics Committees and with informed consent. Ovarian specimens were obtained in the course of standard debulking surgery at Hammersmith Hospitals Trust, London, United Kingdom. All other specimens were obtained during standard surgery at the Veterans Affairs Palo Alto Health Care System and Stanford University School of Medicine, Palo Alto, California. A total of 41 specimens were obtained from patients diagnosed with ovarian carcinomas (n = 11), colorectal carcinomas including benign polyps and liver metastasis (n = 21), esophageal-gastric carcinomas (n = 3), pancreatic ductal carcinomas (n = 3), and mammary epithelial tumors (n = 3). When feasible, specimens from adherent normal tissue and from metastatic secondary tumors were included in the studies.

Preparation of Tissue Explants and Culture Conditions. Immediately after surgical removal the tissue specimens (<1 cm³) were placed on ice in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% FCS (Invitrogen, Carlsbad, CA) and 100 units/mL penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO). Tissue samples were dissected on ice and homogeneous areas selected for each experiment. By dicing the specimens into crossed surgical blocks, cubes of <1 mm³ were prepared, rinsed, and placed in 24-well plates in IMDM supplemented with 5% FCS, 4 μg/mL transferrin, 10 μmol/L insulin, 1 μmol/L hydrocortisone, 10 nmol/L epidermal growth factor, 10 nmol/L transforming growth factor α, and 1 μmol/L estradiol (Sigma-Aldrich). Alternatively, specimens (colorectal and mammary carcinomas) were placed in Millicell 0.45-μm membrane culture plates (Millipore, Billerica, MA) and incubated in the culture media described above. Tissue specimens were incubated at 37°C in an atmosphere of 5% CO₂ for the duration of the study.

Tissue Infection and Harvesting. Each virus was added at 1 × 10⁶ particles/mL medium to each tissue sample in 24- or 6-well dishes. Virus was removed after 2 hours and fresh medium added to the tissue. The following adenoviruses were used: wild-type 5 (Ad5); dl922-947, a mutant with the CR2 region deleted defective in pRb binding (34); and dl312, a replication-defective
mutant with the entire E1A region deleted (42). Tissue incubations were stopped after 1.5 to 48 hours in culture by washing tissue sections in PBS prior to fixation and processing for immunohistochemical analysis. In some studies tissue samples were kept in culture up to 96 hours. Medium was changed every 24 to 48 hours.

**Fixation of Tissue.** Tissue samples were fixed in 4% formalin (PBS buffered) at 4°C for 48 hours, dehydrated in increasing concentrations of ethanol (70% to 100%), and processed for paraffin embedding and sectioning. For frozen sections, tissue samples were placed in the ornithine carbamyl transferase compound (Sakura Finetek, Zoeterwoude, the Netherlands) and frozen at −80°C prior to sectioning.

**General Histopathology and Immunohistochemistry.** Evaluation of tissue survival, cytopathic effect, and general tissue/cell morphology was done by examination of H&E-stained paraffin sections according to standard procedures. The fraction of proliferating cells in each tissue sample was estimated on sections stained for proliferating cell nuclear antigen (PCNA) according to the following protocol. The sections were dewaxed in xylene and rehydrated in ethanol of decreasing concentrations. The sections were unmasked with Dako target retrieval solution (Ely, United Kingdom) according to the manufacturer’s protocol, followed by blocking of nonspecific binding with normal rabbit serum for 30 minutes. A mouse monoclonal primary antibody against human PCNA (Dako) was applied at 1:200 dilution in PBS and incubated for 1 hour at 18°C to 25°C. The sections were washed in PBS for 3 × 5 minutes, incubated with a biotin-conjugated rabbit anti-mouse antibody (Dako) for 30 minutes, and washed 2 × 5 minutes in PBS. Tissue sections were treated with 0.03% hydrogen peroxide in methanol for 20 minutes, washed in PBS for 2 × 3 minutes, and incubated with streptavidin-horseradish peroxidase complex (Dako) for 30 minutes. After washing in PBS for 3 × 5 minutes the slides were developed using the substrate 3,3′-diaminobenzidine (Dako) as a chromogen. Development of staining was monitored by microscopy and stopped after 5 to 15 minutes by immersion in distilled water. The sections were counterstained with hematoxylin, dehydrated in increasing concentrations of ethanol, histocleared with xylene, and mounted for light microscopy analysis.

A similar protocol was followed for detection of other cellular and viral proteins. A goat polyclonal hexon-specific anti-Ad5 antibody (Chemicon, Temecula, CA) was used at a dilution of 1:200 to determine viral uptake and assembly. A rabbit polyclonal anti-E1A antiserum (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used at a dilution of 1:50 to determine viral replication and early gene expression. A mouse monoclonal human-specific anti-CAR antibody (American Type Culture Collection, Manassas, VA), used at 1:1000 for detection of cellular CAR. A mouse monoclonal anti-human αv, integrin antibody (Chemicon) was used at 1:1000 for detection of αvβ3 and αvβ5 integrins. Secondary antibody incubation and detection with 3,3′-diaminobenzidine was as described above. Staining for E1A, CAR, and integrins was done on frozen tissue sections and for hexon on paraffin-embedded tissue.

**Quantitation of Cytopathic Effect, PCNA, Hexon, and E1A Staining by Immunohistochemistry.** Scoring of staining for each treatment group and each case was done on three to six sections per treatment group. The number of positive cells was counted and compared with the total number of live epithelial cells in each section (a total of 1,000 cells per section was counted). For quantitation of virus-induced cytopathic effect and PCNA-positive cells, the proportions of cells demonstrating CPE or staining were expressed as percentages after averaging all slides in each treatment group from an individual case. For hexon and E1A staining the following criteria were used to quantitate the ratios of positively stained cells: −, no staining; +, <20% of cells positive; ++, 20% to 40% of cells positive; and +++, >40% positive. Statistical analysis was by t test, two-tailed ANOVA.

**Viral Replication.** After the initial 2-hour infection, the tissue was rinsed and fresh medium added to each culture well. At specific time points, 2 to 5 days postinfection, media alone or tissue and medium were collected, freeze-thawed thrice, serially diluted, and titered on HEK293 cells by the limiting dilution method (determination of 50% tissue culture infective dose (TC ID50)) as previously described (43). The plates were incubated at 37°C and cytopathic effect determined 10 days postinfection. Each sample was determined in duplicates or triplicates in the TC ID50 assay, expressed as infectious units per milliliter and averaged and presented as the ratio of each replication-competent virus (Ad5 and d922-947) to the replication defective mutant (dl312). Statistical analysis was by t test, two-tailed ANOVA.

**RESULTS**

**Tissue Survival.** Forty-one tumor specimens ranging from benign cysts to malignant adenocarcinomas derived from 31 patients were processed for the study. The tumors included 16 primary colorectal adenocarcinomas, 3 benign colorectal polyps, 2 liver metastatic carcinomas from colorectal cancers, 3 esophageal-gastric adenocarcinomas, 3 pancreatic ductal carcinomas, 3 mammary adenocarcinomas, 5 primary ovarian adenocarcinomas with 2 benign cysts, 2 secondary ovarian tumors from omental tissue, 1 peritoneal mesothelioma, and 1 borderline ovarian tumor (selected specimens in Table 1). Initially, procedures to enable tissue survival in culture for 24 to 96 hours were established by testing nutrient-rich culture media supplemented with growth factors. Tissue survival was enhanced when growth factors such as insulin and epidermal growth factor were included and when IMDM medium was used rather than several other media formulations (data not shown). The addition of insulin was essential for survival of all explants for more than 24 hours. Tissue viability was also dependent on the specific cell type and morphology of each tumor. Adenocarcinomas showed the best survival rates with a high fraction of live cells and intact tissue morphology for up to 48 hours in culture. Benign tumor specimens and normal tissue could only be kept viable ex vivo for 24 hours. Representative results from primary mammary, colorectal, rectal, and ovarian adenocarcinomas kept in culture for 20 to 40 hours are showed in Fig. 1. Tissue samples from the same patient specimen were incubated up to 40 hours in culture media followed by H&E and PCNA staining after 24 hours or 20 and 40 hours in culture (Fig. 1A-C and D-E, respectively). Tissue specimens showed intact morphology.
with well-defined, differentiated epithelial cells during the entire 40-hour incubation period represented by a primary ovarian papillary adenocarcinoma (Fig. 1D, E). The viability of the tissue was verified by PCNA staining of the same tissue samples demonstrating the presence of numerous proliferating cells at each time point (Fig. 1A-E). The ratio of proliferating epithelial cells staining positive for PCNA to the total epithelial cell population were estimated to be 40% in the mammary, 45% in the colorectal, and 40% in the rectal carcinoma tissues after 24 hours ex vivo (Fig. 1A-C). When the same samples were evaluated for ratio of proliferating cells to total tumor cells only, the PCNA-positive fraction was 80% to 85% in all tested specimens (Fig. 1A-C). Analysis of cell viability over time was done on four of the ovarian adenocarcinomas, demonstrating that viability and cell death were highly variable dependent on the characteristics of each specific tissue (selected specimens in Table 2). Factors such as tissue structure, degree of tumor necrosis in situ, morphology, and degree of cell differentiation affected tissue viability ex vivo. The degree of PCNA-positive cells at baseline varied from 5% to 50% of the total epithelial cell population but remained at the same level in each tissue for the entire 40- to 48-hour culture period in all tested samples (Fig. 1A-C). When adenocarcinoma explants were kept in culture for more than 48 hours a gradual decrease in the number of live cells was observed; after 96 hours <5% live epithelial cells and no proliferating PCNA-positive cells were detectable (data not shown). The gradual deterioration of adenocarcinoma tissue after 48 hours and of benign/normal tissue after 24 hours in culture was independent of growth factor supplementation and media formulations. Therefore, in subsequent studies we focused on culturing of adenocarcinoma tissue for no more than 48 hours to enable evaluation of viral infectivity and replication.

**Viral Infectivity of Tumor Tissue Ex vivo.** To determine whether the tumor sections could be evaluated for adenoviral infectivity and replication under the ex vivo conditions, samples were infected with replication-competent (Ad5, dl922-947) and replication-defective viruses (dl312). Initially, infectivity and replication were determined in the ovarian tissue specimens by the detection of Ad5 hexon protein at different periods, 1.5 to 46 hours postinfection. All primary ovarian adenocarcinomas were positive for viral uptake as determined by Ad5 hexon staining from 24 to 48 hours postinfection (Table 1). A representative tissue sample is shown in Fig. 2, a malignant papillary serous cystadenocarcinoma (all sections from specimen 3). This tissue showed high levels of hexon expression at 17 hours, 20 hours, and 40 hours postinfection but not after 1.5 hours. The dl922-947 mutant seemed to infect and/or replicate more efficiently than wild-type virus determined at 2, 17, and 40 hours postinfection with highest levels at 40 hours (>50% positive epithelial cells), indicating replication rather than uptake (Fig. 2). The intensity of the hexon staining for the non-replicating control virus dl312 was low, with <5% of total cells positive and no change over time, demonstrating uptake of virus rather than replication as expected. Uninfected tissue was not positive for hexon (not shown).

**Viral Replication in Tissue Ex vivo.** To further determine if virus could replicate in tumors sensitive to viral infection, the presence of E1A in infectible specimens was determined. The ovarian adenocarcinomas with the highest levels of hexon postinfection also had detectable E1A levels (Table 1). The E1A expression paralleled the observations for hexon expression with the highest levels in tissue infected with the dl922-947 mutant and no detection of E1A expression in tissue infected with the nonreplicating dl312 mutant. Representative E1A staining is shown in a primary ovarian serous adenocarcinoma (specimen 9a) 25 hours postinfection (Fig. 3A).

### Table 1  Infected (Ad5) and uninfected tumor specimens examined for morphologic changes, cytopathic effect, and viral gene expression

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Tumor differentiation</th>
<th>IHC staining</th>
<th>Ex vivo survival (h)</th>
<th>Hexon*</th>
<th>E1A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9a. Serous adenocarcinoma</td>
<td>Well</td>
<td></td>
<td>46</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>9b. Omental metastasis (from 9a)</td>
<td>Well</td>
<td></td>
<td>46</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>3. Serous papillary cystadenocarcinoma</td>
<td>Well</td>
<td></td>
<td>40</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>1a. Malignant mesothelioma, primary peritoneal</td>
<td>Well</td>
<td></td>
<td>36 (48);†</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6b. Benign ovarian cysts</td>
<td>Well</td>
<td></td>
<td>26</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8. Invasive adenocarcinoma</td>
<td>Moderate</td>
<td></td>
<td>48 (96);†</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>6a. Serous adenocarcinoma</td>
<td>Moderate</td>
<td></td>
<td>48</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>10. Adenocarcinoma, omental metastasis</td>
<td>Poor</td>
<td></td>
<td>48</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>7. Borderline mucinous tumor</td>
<td>Poor</td>
<td></td>
<td>36 (96);†</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>4. Serous adenocarcinoma</td>
<td>Poor</td>
<td></td>
<td>26 (49);†</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>1b. Ovarian carcinoma, metastasis from primary mesothelioma (from 1a)</td>
<td>Poor</td>
<td></td>
<td>24 (48);†</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26. Colorectal adenocarcinoma</td>
<td>Well</td>
<td></td>
<td>48</td>
<td>ND</td>
<td>++</td>
</tr>
<tr>
<td>32. Breast adenocarcinoma</td>
<td>Well</td>
<td></td>
<td>48</td>
<td>ND</td>
<td>++</td>
</tr>
<tr>
<td>34. Colorectal adenocarcinoma</td>
<td>Well</td>
<td></td>
<td>48</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>35. Rectal adenocarcinoma</td>
<td>Well</td>
<td></td>
<td>48</td>
<td>ND</td>
<td>++</td>
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</table>

**NOTE:** Abbreviations: IHC, immunohistochemistry; ND, not determined.

*Cells were determined hexon-positive for intracellular staining only: -, no staining of epithelial cells; +, <20% positive epithelial cells; ++, 20% to 40% positive epithelial cells; ++++, >40% positive cells.

Specimens with the same number are from the same patient.

Gradual decrease in PCNA positive cells with <5% at the indicated time.
Early viral gene expression was also confirmed in the corresponding metastatic omental implants (specimen 9b) at 25 hours and 40 hours postinfection and was higher in the primary tumor tissue, as expected from infectivity data with higher hexon staining in the primary tumor (Fig. 3A; Table 1).

A series of adenocarcinomas originating from various tissues including pancreas, colon, rectum, gastric, and mammary tissues was tested for early viral gene expression as an indication of infectibility and potential viral replication. A selection of tissue samples was tested for both E1A expression and production of replicating units by the TC ID50 assay (Table 3). The majority (12 of 15) of colorectal adenocarcinomas supported E1A expression and 11 of these also supported replication. These findings were similar in most other tumor specimens, for example, mammary and gastric tumors (data not shown). Representative sections from mammary, colorectal, and rectal tumors infected ex vivo are shown in Fig. 3B to D.

The E1A expression levels were highest in all tissues infected with the dl922-947 virus and as expected no expression was detected in samples infected with dl312 (Fig. 3A-D). The E1A expression remained at the same levels from 24 to 40 hours postinfection (data not shown). Individual bursting cells could be observed in both wild-type and dl922-947-infected samples (Fig. 3E). The ovarian specimens with highest levels of E1A expression also showed the highest levels of PCNA expression and cytopathic effect (Tables 1 and 2, cases 3 and 9). Both specimens infected with replication-competent and replication-defective (dl312) viruses as well as uninfected tissue showed cytopathic effect, with a trend toward higher levels of cytopathic effect in the dl922-infected samples. However, the increase was only significant in the dl922-infected samples compared with the uninfected but not to dl312-infected tissue (cases 3 and 9). There was no significant difference in cytopathic effect between dl312-infected and uninfected specimens. Whereas cytopathic effect is a rather insensitive measurement of virus-induced cell death at early time points (20–48 hours postinfection), PCNA staining is a more direct and sensitive measure of cell viability and virus-induced S-phase entry in tumor cells. Highly increased PCNA levels were observed in the same specimens 20 to 48 hours post viral infection (Table 2). Infection with the replication-defective virus did not result in significant changes in PCNA levels compared with uninfected cells, whereas the dl922-infected tissue showed significantly higher staining than both dl312-infected and uninfected specimens at both time points (Table 2). Taken together, these data indicate that both wild-type virus and the Rb-binding–defective replication-selective mutant could replicate and promote S-phase entry in tumor cells ex vivo.

To further verify that the tumor specimens could support viral replication, tissue and incubation media were harvested for determination of viral replication 2 to 5 days postinfection. Five tumor specimens that had previously been shown to express E1A postinfection were analyzed for recovery of viral units (Table 3). In parallel, the corresponding normal tissue specimens (when available) were analyzed for viral replication under similar conditions. All tumor samples supported viral replication of both Ad5 wild-type and dl922 viruses in the range of $1 \times 10^5$ to $1 \times 10^6$ infectious units/mL (recovered from one to five tissue samples ~1 mm$^3$), whereas no replication was observed in tissue infected with the nonreplicating dl312 mutant (basal level postinfection $1 \times 10^2$ to $1 \times 10^5$ infectious units/mL). Significantly higher replication was seen in all tumor specimens compared with normal tissue for both viruses. Four of five tumor samples infected with the dl922 mutant showed higher levels of replication than when infected with Ad5 wild-type virus with no differences between viruses in one specimen (Table 3). The normal corresponding tissue cubes displayed lower levels of replication, and in three of four specimens wild-type virus expression remained at the same levels from 24 to 40 hours postinfection (data not shown). Individual bursting cells could be observed in both wild-type and dl922-947-infected samples (Fig. 3E). The ovarian specimens with highest levels of E1A expression also showed the highest levels of PCNA expression and cytopathic effect (Tables 1 and 2, cases 3 and 9).

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replicated significantly better than the dl922 mutant. However, viral replication seemed not to be supported in all infectible specimens as determined by hexon and E1A staining of consecutive sections of each tissue and by TC ID₅₀ infectious unit assay (Table 1 and data not shown). Whether viral replication could be supported in each case was likely due to differences in tissue morphology and gene mutations/viral replication seemed not to be supported in all infectible specimens as determined by hexon and E1A staining of consecutive sections of each tissue and by TC ID₅₀ infectious unit assay (Table 1 and data not shown). Whether viral replication could be supported in each case was likely due to differences in tissue morphology and gene mutations/viral replication. These factors are known to affect viral replication, illustrated by the well-differentiated ovarian adenocarcinomas supporting viral replication (Table 3) as opposed to the poorly differentiated cases not supporting E1A expression and replication (Table 1).

**Presence of CAR and α₅ Integrins.** The major mechanisms for adenoviral cell entry have been reported to occur through receptor-mediated processes involving CAR, α₅β₃, and α₁β₃ integrin receptors (44, 45). Low infection levels have previously been observed in several ovarian cancer cell lines and seemed to correlate with low levels or absence of CAR, α₁β₃, or α₁β₃ integrin expression (46–48). To determine if viral uptake could be correlated with the presence of these receptor proteins in the explant specimens, the samples were analyzed by immunohistochemistry. CAR could not be detected in any of the tested samples (n = 0/4), whereas α₅ integrins were present in both primary ovarian tumors and the omental implants (cases 9 and 10) (n = 6/6). No correlation with viral infectivity could be determined in this small sample set due to limitations in tissue availability.

**DISCUSSION**

Virotherapy holds great promise as a treatment platform to improve prognosis in patients with advanced or recurrent adenocarcinomas. One of its advantages is the lack of cross-resistance with the standard therapies used for adjuvant treatments in most cancers, such as chemo- or radiotherapy. A suitable experimental model for immediate evaluation of antitumor efficacy of viral mutants in individual human tumor samples has not been available thus far. Tumor cell lines are often used as the standard test system for viral efficacy, but cancer cell lines passaged in vitro for years might not reflect the biology of tumors in vivo. Cell lines often lose essential cell surface proteins and gain gene mutations when grown in culture. For example, the adenoviral receptor proteins CAR and heparan sulfate glycosaminoglycan expression were shown at different levels when the same cell line was cultured under various conditions, previously shown for several cellular proteins (49–54). In addition, gene mutations and homozygous deletions have been found to be less common in ovarian tumors in situ than in cancer cell lines (39, 50, 51). Dissociation of epithelial tissue followed by culturing of cells is another commonly

<table>
<thead>
<tr>
<th>Case, papillary adenocarcinoma</th>
<th>Cytopathic effect (% of total epithelial cells)</th>
<th>PCNA staining (% of total epithelial cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(dl312)</td>
<td>(dl312): 9 ± 2, 43 ± 6, 30 ± 10</td>
<td>(dl312): 5 ± 1, 6 ± 2, 5 ± 1</td>
</tr>
<tr>
<td>Case 9, serous adenocarcinoma</td>
<td>Uninfected</td>
<td>Infected</td>
</tr>
<tr>
<td>(dl922-947)</td>
<td>9 ± 2, 43 ± 6, 30 ± 10</td>
<td>(dl922-947): 40 ± 10, ND</td>
</tr>
<tr>
<td>(dl312)</td>
<td>(dl312): 9 ± 2, 43 ± 6, 30 ± 10</td>
<td>(dl312): 5 ± 3, ND</td>
</tr>
</tbody>
</table>

*P < 0.05, dl922-947-infected specimens compared with uninfected tissue.

†P < 0.05, dl922-947-infected specimens compared with dl312 treated and uninfected tissue.

![Fig. 2](image_url) Ovarian adenocarcinoma tissue (specimen 3) infected with Ad5 wild-type, dl922-947, and dl312 mutant viruses. Infectivity was determined 40 hours postinfection by staining for intracellular hexon expression. Intranuclear hexon staining was observed in all specimens. The hexon levels were highest in the dl922-947-infected tissue with >50% of epithelial cells positive (right), slightly lower (40-50%) in the wild-type virus–infected sample (left), and <5% in the dl312-infected tissue (middle). Original magnification ×400.
Adenocarcinoma tissue infected with Ad5 wild-type, dl922-947, and dl312 mutant viruses to determine E1A expression as an indication of replication 25 hours postinfection in culture. A, ovarian serous adenocarcinoma (specimen 9a) B, mammary epithelial carcinoma (specimen 32) C, colorectal carcinoma (specimen 26), and D, rectal carcinoma (specimen 35) stained with an anti-E1A antibody on frozen sections. Intranuclear E1A expression was identified in Ad5 (left) and dl922-947-infected tissue (right) but not in tissue infected with the nonreplicating dl312 control virus (middle). When specimens were infected with Ad5, 20% to 40% of total epithelial cells were positive for E1A, whereas tissue infected with the dl922-947 mutant showed slightly higher E1A staining (>40%). E, individual bursting cells could be detected throughout tissue infected with replication-competent viruses as identified with anti-hexon staining 40 hours postinfection of an ovarian adenocarcinoma infected with dl922-947. Original magnification ×400 (A-D) and ×600 (E).
used in vitro technique. Mechanical and enzymatic cell dispersion will result in loss of cell polarity, changes in protein expression patterns and loss of tissue structure, and so might not reflect the cells in vivo with regard to viral infection/replication. Several animal models are also being used for determination of efficacy of both chemotherapy and oncolytic viruses with studies primarily done in nude mice with human tumor xenografts derived from tumor cell lines (29, 40, 41). Few studies have been in animals with intact immune system to determine efficacy of oncolytic viruses in vivo with regard to viral clearance or efficacy. We recently developed murine tumor models to evaluate the contribution of the host immune response to antitumor efficacy and cytotoxicity in the presence of cytokines and other immune factors to more accurately reflect the clinical situation (43, 55). Whereas both the in vivo and in vitro models are essential for evaluation of safety and antitumor efficacy and selectivity, limited data can be obtained from such studies regarding viral potency in a specific type of tumor tissue. Therefore, conditions were developed that could support small samples of intact adenocarcinoma tissue in culture for rapid evaluation of viral infectivity and selective replication in each patient case. In future studies, both design and selection of novel mutants could be optimized prior to clinical testing using similar ex vivo culture conditions. Identification of predictive factors for tumor response to viral mutants might facilitate the targeting of specific patient groups resulting in better efficacy. Ex vivo analysis of patient tissue would also enable assessment of correlation of tumor infectivity with receptor expression and replication with alterations in cell cycle regulation, CAR and α, integrins, or p53/pRb/p16, respectively.

The findings presented in this report demonstrate that small specimens of adenocarcinomas originating from various tissues could be viably maintained ex vivo for evaluation of tissue susceptibility to adenoviral infection and ability to support viral replication. In a recent study, a perfusion system was used to infect liver wedges ex vivo with another oncolytic virus, herpes simplex virus (56). Although the authors showed good replication after 48 hours, limited data were available on tissue viability and viral gene expression. The perfusion system described is rather complex and requires both larger tissue samples and cannulation of small blood vessels. In contrast to herpes simplex virus, the time for adenoviral replication is significantly longer (~18-24 hours) necessitating more focus on gene expression analysis than viral replication at early time points. In another recent publication it was reported that tissue from Barret’s esophagus could be kept in culture ex vivo for 24 hours (57). During this time frame, it was shown that the tissue could be infected by a nonreplicating green fluorescent protein-expressing virus, whereas viral replication and oncolysis was not addressed. However, for evaluation of potency of oncolytic adenoviral mutants it is essential to determine the level of viral gene expression and/or replication and tissue spread in each case as described here. Our data show that small sections of intact adenocarcinoma tissue could be kept in culture for at least 46 hours by supplementation of growth factors in nutrient-rich media. During this incubation period, tumor tissue could be evaluated for uptake of viral mutants determined by late viral gene expression. After 20 to 46 hours, tumor tissue could also be evaluated for viral replication by early gene expression and by recovery of infectious units for quantification. In this system, adenocarcinoma specimens could be maintained ex vivo with adequate viability whereas benign cystic and normal tissue quickly deteriorated with no live cells beyond 24 hours. Despite careful sampling and selection of homologous tumors we observed a high degree of cell heterogeneity, both in tumors derived from different patients as well as in metastatic tumors in the same patient. Consequently, the sensitivity to viral infection and the ability to support viral replication was highly variable. In some cases, the majority of epithelial cells showed intracellular hexon expression, whereas in other cases only a few viral particles could be detected intracellularly. Other tissue specimens showed an accumulation of viral particles along the cell surface with few particles entering the cells. One reason for the observed variability in both viral uptake and replication could be due to differences in protein expression patterns of each tumor specimen. For example, the level of expression of viral receptors CAR, αβ3, and αβ5 integrins would be expected to influence cellular uptake of virus. Although the major adenoviral receptor CAR could not be detected in infected samples, αβ integrins were present in the same samples. Adenovirus has been proven to enter epithelial cells efficiently through interaction of the penton protein with CAR facilitates the internalization but is not required (44, 45, 52). Additional cellular proteins such as a heparan sulfate receptor (heparan sulfate glycosaminoglycan) and the αβ integrin have been implicated in viral binding and cell entry (53, 54, 58). However, it is unclear whether one or several of these receptor proteins or as yet unidentified cellular factors are essential for efficient viral infection (59). A larger tumor sample set would be needed for correlation of the presence of cell surface receptors with viral infectivity and replication in tumor specimens. Other factors that were likely to influence viral infectivity of the

### Table 3: Replication of wild-type Ad5 and dl922-947 correlates with E1A expression in tumor specimens

<table>
<thead>
<tr>
<th>Adenocarcinoma tissue</th>
<th>E1A (IHC)</th>
<th>Tumor (ratio IU/mL)</th>
<th>Normal (ratio IU/mL)</th>
<th>Tumor/normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 26, colorectal</td>
<td>+</td>
<td>9.1 × 10^3</td>
<td>1.2 × 10^4</td>
<td>670†</td>
</tr>
<tr>
<td>Case 32, mammary</td>
<td>+</td>
<td>1.1 × 10^4</td>
<td>9.2 × 10^4</td>
<td>2.0 × 10^3</td>
</tr>
<tr>
<td>Case 34, colorectal</td>
<td>+</td>
<td>4.3 × 10^4</td>
<td>3.0 × 10^3</td>
<td>520†</td>
</tr>
<tr>
<td>Case 35, rectal</td>
<td>+</td>
<td>3.5 × 10^6</td>
<td>4.0 × 10^4</td>
<td>1.7 × 10^5</td>
</tr>
<tr>
<td>Case 9, ovarian serous</td>
<td>+</td>
<td>1.2 × 10^5</td>
<td>3.3 × 10^5</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE: Abbreviations: IU/mL, infectious units/mL; +, positive E1A staining in tumor tissue (refer to Table 1).  
*P < 0.05, for ratio of Ad5 or dl922-947 to dl312 in tumor compared with corresponding ratio in normal tissue.  
†P < 0.05 for ratio of Ad5 or dl922-947 to dl312 in tumor tissue being higher than the corresponding Ad5 ratio.  
‡P < 0.05, for ratio of Ad5 to dl312 in normal tissue being higher than the corresponding dl922-947 ratio.
explants were physical barriers such as connective tissue and mucinous secretions. Stromal sections with dense connective tissue and mucus production were frequently observed in certain areas of tumor samples. The degree of tumor differentiation in each tumor in situ also influenced the ability of virus to infect and spread efficiently in the tumor samples ex vivo.

Despite the presence of a time-dependent increase of Ad5 hexon expression in 8 of 11 ovarian adenocarcinomas only 3 of these specimens showed E1A expression, 2 specimens with high levels and 1 with lower levels (Table 1). One possibility is that E1A was expressed at low levels in some tumors but could not be detected in the assay, immunohistochemistry optimization being limited by tissue availability in most of the ovarian cases. However, E1A expression was present in the majority of cases tested from colorectal and mammary epithelial tumors. The E1A expression seemed stable from 25 to 46 hours in all tested tissue and mucus production were frequently observed in certain mucinous secretions. Stromal sections with dense connective tissue and mucus production were frequently observed in certain areas of tumor samples. The degree of tumor differentiation in each tumor in situ also influenced the ability of virus to infect and spread efficiently in the tumor samples ex vivo.

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