Abstract

We studied the susceptibility of primary ovarian cancer cells to oncolytic adenoviruses. Using gene expression profiling of cancer cells either resistant or susceptible to viral oncolysis, we discovered that the epithelial phenotype of ovarian cancer presents a barrier to infection by commonly used oncolytic adenoviruses targeted to coxsackie-adenovirus receptor or CD46. Specifically, we found that these adenovirus receptors were trapped in tight junctions and not accessible for virus binding. Accessibility to viral receptors was critically linked to depolarization and the loss of tight and adherens junctions, both hallmarks of epithelial-to-mesenchymal transition (EMT). We showed that specific, thus far little-explored adenovirus serotypes (Ad3, Ad7, Ad11, and Ad14) that use receptor(s) other than coxsackie-adenovirus receptor and CD46 were able to trigger EMT in epithelial ovarian cancer cells and cause efficient oncolysis. Our studies on ovarian cancer cultures and xenografts also revealed several interesting cancer cell biology features. Tumors in situ as well as tumor xenografts in mice mostly contained epithelial cells and cells that were in a hybrid stage where they expressed both epithelial and mesenchymal markers (epithelial/mesenchymal cells). These epithelial/mesenchymal cells are the only xenograft-derived cells that can be cultured and with passaging undergo EMT and differentiate into mesenchymal cells. Our study provides a venue for improved virotherapy of cancer as well as new insights into cancer cell biology. [Cancer Res 2009;69(12):5115–25]

Introduction

Cancer derived from ovarian surface epithelium accounts for >90% of ovarian cancer. Generally, epithelial cells are characterized by polarized membranes and tight junctions that seal the paracellular space. During progression toward metastatic disease, epithelial cancers undergo an epithelial-to-mesenchymal transition (EMT), a cellular transdifferentiation program where epithelial cells lose characteristic features, such as tight and adherens junctions, and gain properties of mesenchymal cells (1). EMT, as well as a high mutation rate and epigenetic instability of cancer cells, contributes to genetic and phenotype heterogeneity of tumor cells present in a given tumor (2). We hypothesized that the phenotypic plasticity of ovarian cancer cells results in different susceptibility of malignant cell subsets to antitumor agents. Specifically, we focused our studies on susceptibility of ovarian cancer cells to oncolytic adenoviruses, e.g., viruses that are capable of amplifying the input dose through replication in a tumor-dependent fashion. For humans, 51 different adenovirus serotypes have been identified and classified into six species (A-F). Thus far, all oncolytic adenoviruses used clinically were based on species C serotype 5. Although oncolytic adenoviruses have been proven safe in patients, they have fallen short of their expected therapeutic value as monotherapies (3). Species A and C to F use the coxsackie-adenovirus receptor (CAR) as a cellular receptor, whereas species B adenoviruses use other attachment receptors, including CD46 and a yet nonidentified receptor(s), which is referred to as receptor X (4). CD46 is one of the few examples of non-CAR receptors that are used by oncolytic adenoviruses to infect their target cells. Specific subspecies of human adenoviruses that use receptor(s) other than CAR and CD46 should focus on nonepithelial malignancies and (b) controlled induction of EMT in epithelial ovarian cancer cells can sensitize them to viral oncolysis. In this context, we show that a specific subspecies of human adenoviruses that uses receptor(s) different from CAR and CD46 is efficient in killing epithelial ovarian cancer cells, mainly by forcing these cells into EMT.

Materials and Methods

Tumor cell culture. Tumor tissue from biopsies was digested with proteases and cells were cultured as described in Supplementary Materials. Xenografts were established by injecting cancer cells in Matrigel into the mammary fat pad of CB17 SCID-beige mice.

Adenoviruses. The following adenovirus vectors have been described previously: Ad5/35.IR-E1A/TRAIl, Ad5.IR-E1A/TRAIl (6), Ad5/35Δ24.Ki. COX (7), Ad5-GFP, Ad5/35-GFP, and Ad5/35-β-galactosidase (β-gal; ref. 8). The wild-type adenoviruses Ad3 (GB strain), Ad7p (Gomen stain), Ad11p (Slobitski strain), Ad14 (DeWit strain), and Ad35 (Holden strain) were all obtained from the American Type Culture Collection.
The adenoviruses were propagated in 293 cells, CsCl purified, and titered for genomes and plaque-forming units (pfu) as described previously (8).

**Antibodies and inhibitors.** A complete list of all antibodies and inhibitors is provided in Supplementary Materials. Adenoviruses or the adenovirus hexon antibody were labeled with Cy3 using the Cy3 bis-Reactive Dye according to the manufacturer’s instructions (Amersham).

**Animal studies.** All experiments involving animals were conducted in accordance with the institutional guidelines set forth by the University of Washington. To establish subcutaneous tumors, CB17 SCID-beige mice were injected into the mammary fat pad with \( 1 \times 10^5 \) tumor cells. Adenovirus vectors were injected when tumors reached a diameter of 5 mm. To establish mouse models with liver metastases, animals were infused with \( 2 \times 10^6 \) of human tumor cells through a permanently placed portal vein catheter (9). For intravenous application, adenovirus vectors in 100 µL PBS were injected through the tail vein.

The following methods are described in Supplementary Materials: viral attachment/uptake assays, cytosis assays, expression arrays, quantitative reverse transcription-PCR and quantitative PCR analyses, immunofluorescence/confocal analyses, Western blotting, and statistical analyses.

**Results**

**Resistant ovarian cancer cells have an epithelial phenotype.** We subjected primary cultures from tumor biopsies of III and IV ovarian cancer patients to limited dilution culturing and established >100 clonal cultures from each primary culture. Most of the data shown in this study were obtained with clonal cultures derived from biopsy ovc316 obtained from a stage IVB serous ovarian cancer that was resistant to primary chemotherapy. We have similar findings with clonal cultures from other chemotherapy-resistant cancer. The morphology of clonal cultures from ovc316 varied greatly (Supplementary Fig. S1). In a first study, we used Ad5/35.IR-E1A/TRAIL (6) as a model oncolytic adenovirus. This vector is targeted to CD46 by the adenovirus serotype 35 fiber and allows for tumor-specific, replication-activated expression of E1A and TRAIL and efficient tumor cell killing. In cytology studies, we found that the morphologic heterogeneity seen in clonal cultures was also reflected in a heterogenic response to the infection by Ad5/35.IR-E1A/TRAIL (Fig. 1A). Clonal cultures that were resistant to Ad5/35.IR-E1A/TRAIL lysis (\( n = 15 \)) were subjected to genome-wide mRNA expression analysis in comparison with clonal cultures susceptible to viral oncolysis (\( n = 16 \)). We found 983 differentially expressed genes (\( P < 0.017 \)). Hierarchical clustering of these genes showed a clear-cut separation of resistant and susceptible clones (Supplementary Fig. S2). Using gene ontology software (10), we found that pathways involving tight and adherens junction formation and cell adhesion were significantly different in resistant cells (\( P < 0.008 \); Supplementary Fig. S3A-E). Altered RNA expression levels of key genes found in microarray studies were validated by quantitative reverse transcription-PCR (Supplementary Fig. S4). Gene expression on protein level was studied by immunofluorescence and flow cytometry analyses (Fig. 1C and D). Immunofluorescence analyses showed high levels of adherens proteins (E-cadherin), tight junction proteins (occludin and claudins 1-4 and 7), and the epithelial marker EpCAM in resistant cells (Fig. 1D, top; Supplementary Fig. S5). Conversely, susceptible cells predominantly expressed markers that are characteristic for mesenchymal cells (vimentin, laminin, collagen IV, fibronectin, and N-cadherin; Fig. 1D, bottom; Supplementary Fig. S5). Furthermore, expression of CD44 was higher in susceptible than resistant clones. Notably, CD44 is considered a marker for mesenchymal stem cells (11–13). Of particular interest was the high prominence of p120 catenin containing the regulatory NH2 terminus in susceptible cells (N-p120), which, in part, explains why E-cadherin is absent on the surface of these cells (ref. 14; Supplementary Fig. S6). Other marker proteins that discriminated resistant and susceptible clones included cingulin, vinculin, the cytoskeleton protein F-actin, and netrin 4 (Supplementary Fig. S5). Flow cytometry analyses corroborated that clonal ovarian cancer cultures express both epithelial and mesenchymal markers, whereby clones resistant or susceptible to Ad5/35.IR-E1A/TRAIL show the balance greatly shifted toward epithelial or mesenchymal markers, respectively (Fig. 1C). In all studies described below, resistant and susceptible clones were designated as resistant/epithelial (R/E) and susceptible/mesenchymal (S/M), respectively.

We next tested whether our findings could be validated in the primary (low-passage) ovc316 culture from which the clones were derived. We found that E-cadherin-positive and laminin-negative cells were resistant to lysis by Ad5/35.IR-E1A/TRAIL (Fig. 2A). At day 4 after infection, most susceptible cells (laminin- and N-p120-positive) showed bright-red viral hexon signals, indicating viral replication. At day 8 post-infection, only E-cadherin-positive cells remained in infected ovc316 cultures. Flow cytometry analyses revealed that the percentage of vimentin\(^{\text{high}}\) (mesenchymal) cells decreased over time, whereas the percentage of E-cadherin\(^{\text{high}}\) cells increased (Fig. 2B). Notably, E-cadherin\(^{\text{high}}\)/vimentin\(^{\text{low}}\) cells appeared to be more resistant than E-cadherin\(^{\text{high}}\)/vimentin\(^{\text{high}}\) cells. Greater resistance of cells that differentiated toward epithelial cells is also seen in flow cytometry analysis of E-cadherin/N-p120 and EpCAM/CD44.

**Adenovirus receptors are trapped within tight junctions of epithelial ovarian cancer cells.** To study the mechanisms of resistance to killing by Ad5/35.IR-E1A/TRAIL, we first analyzed attachment of \(^{3}H\)-labeled Ad5/35.IR-E1A/TRAIL particles. We found that \( >3 \) times more adenovirus particles attached to S/M than to R/E cells (Fig. 3A, left). Consequently, subsequent infection steps, including virus genome replication and gene expression, were affected in R/E cells (Fig. 3A, middle and right; Supplementary Fig. S7A). Using fluorophore-labeled Ad5/35 particles, we showed inefficient attachment to R/E cells also in
Figure 2. Analysis of primary ovarian cancer culture ovc316 for epithelial and mesenchymal markers after infection with Ad5/35.IR-E1A/TRAIL. A, primary cultures (passage 10) were infected with Ad5/35.IR-E1A/TRAIL at a MOI of 100 pfu/cell and analyzed at days 2, 4, and 8 after infection for immunofluorescence as indicated. Uninfected cells were used as a control. B, flow cytometry of infected cells. Representative samples. Notably, resistant cells that showed cytoplasmic E-cadherin staining in immunofluorescence studies scored negative by flow cytometry analysis for surface E-cadherin.
immunofluorescence studies (Supplementary Fig. S7B). These studies revealed that the post-attachment signaling, which results in reorganization of the F-actin network or recruitment of the focal adhesion protein vinculin in S/M clones, is not activated in R/E clones. Because our data suggested that inefficient attachment of adenovirus particles to epithelial ovarian cancer cells is largely responsible for resistance to killing by Ad5/35.IR-E1A/TRAIL, we concluded that other vectors with an Ad5/35 capsid will also be inefficient in infection/lysis of epithelial ovarian cancer cells. We showed that transduction with an Ad5/35.GFP vector was significantly less efficient in E/R clones than in S/M clones (Supplementary Fig. S8). Overall, the efficiency of Ad5/35.GFP transduction correlated with Ad5/35.IR-E1A/TRAIL-mediated oncolysis when analyzed in individual R/E and S/M clones (Supplementary Fig. S8B).

An oncolytic Ad5/35 vector (Ad5/35Δ24Ki/Cox) expressing the adeno-viruses E1 and E4 gene products under the control of the Ki-67 and the cyclooxxygenase-2 promoter, respectively (7), predominantly killed mesenchymal cells (vimentin<sup>high</sup>/p120<sup>high</sup>), whereas epithelial cells (E-cadherin<sup>high</sup>) were resistant (Supplementary Fig. S8C). However, confocal microscopy revealed that the majority of CD46 signals were inside tight junctions, with few receptor molecules localized to the apical and basolateral membrane of E/R cells. In contrast, in S/M cells, CD46 was evenly distributed over the entire cell membrane. Cy3-labeled adeno-virus particles did not bind to CD46 trapped in tight junctions (Fig. 3C; Supplementary Fig. S10). In contrast, in S/M cells, adeno-virus particles attached to cells from both apical and basolateral sides. In R/E cells, α<sub>V</sub> integrins were found in tight junctions and on the basolateral membrane. Consequently, adeno-virus particles that were attached to the few apically localized CD46 molecules cannot be internalized into R/E cells. In contrast, in S/M cells, both CD46 and α<sub>V</sub> integrin colocalize, conferring efficient adeno-virus internalization. These findings were corroborated by transduction studies in transwell chambers, where Ad5/35.GFP vectors were applied from either the apical or the basolateral sides of cells (Fig. 3D; Supplementary Fig. S8C and D). Although the percentage of GFP-expressing cells was comparable in S/M and ovc316m cells infected from either the apical or the basolateral side, in R/E cells, apical infection was markedly less efficient due to the low numbers of CD46 receptors and the apparent lack of α<sub>V</sub> integrins on the apical side. The finding that basolateral transduction of R/E cells was efficient can be explained by the high density of α<sub>V</sub> integrins and the presence of a few CD46 receptors on this membrane side.

Epithelial phenotype of ovarian cancer cells is a barrier to adenovirus infection in vivo. On tumor sections from ovarian cancer patients, we found large subsets of malignant epithelial cells surrounded by tumor stroma consisting of laminin (Fig. 4A). This morphology could be reproduced in mouse xenografts derived from ovarian cancer cultures. In both patient tumors and xenografts, CD46 and α<sub>V</sub> integrins were colocalized with the tight junction protein claudin 7, supporting our in vitro findings (Fig. 4A; Supplementary Fig. S11A and B). This histology was observed for all 10 analyzed biopsies from ovarian cancer patients (Supplementary Fig. S11C) as well as in xenografts derived from the ovarian cancer cell line SKOV3-ip1 (Supplementary Fig. S11D). As expected from this morphology, both intratumoral and intravenous injection of Ad5/35.IR-E1A/TRAIL into mice bearing subcutaneous ovc316 xenografts had no effect on tumor growth compared with PBS-injected mice (data not shown). No viral replication (based on adeno-virus hexon staining) was detectable in ovc316 tumors at day 8 after intratumoral injection of Ad5/35.IR-E1A/TRAIL (Fig. 4A, bottom). Intratumoral injection of Ad5/35-GFP resulted in very few GFP-expressing cells directly surrounding the needle track. Intravenous injection of an Ad5/35 vector conferred transgene expression only in sparse cells around the tumor periphery, a tumor area that contained blood vessels.

Although the majority of cells in early-passage ovc316 cultures was susceptible to Ad5/35 infection in vitro (see Fig. 2), in vivo transduction after Ad5/35 injection into ovc316 xenografts was very inefficient. To clarify this discrepancy, we performed flow cytometry analyses for EpCAM, vimentin, and CD44 on cell suspensions of ovc316 xenograft tumors and on cultured ovc316 cells from passages 1 and 20 (Fig. 4B). In xenografts, the vast majority of ovarian cancer cells (>80%) expressed high levels of EpCAM, whereby a significant fraction of these cells were also positive for vimentin and CD44, indicating that these cells were in a hybrid E/M stage (E/M cells). Interestingly, most cells isolated from xenografts that adapted to tissue culture were E/M cells. When further passaged, E/M cells lost EpCAM expression and differentiated toward the mesenchymal phenotype. As outlined above, E/M cells with intracellular mesenchymal features and the mesenchymal cells represented the populations susceptible to oncolysis in vitro. Similar to what we observed for ovc316, there was an in vivo and in vitro discrepancy between the phenotypes for ovarian cancer SKOV3-ip1 cells, cervical adenocarcinoma HeLa cells, colon cancer HT-29, and liver endothelial cancer SK-Hep1 cells (Supplementary Fig. S12). Notably, R/E cell clones do not form tumors. Tumor formation requires the presence of E/M hybrid cells, which differentiate in vivo to epithelial cells. In situ, these hybrid cells are embedded in nests of epithelial cancer cells, which protects them from Ad5/35 transduction in vivo.

Epithelial phenotype of ovarian cancer cells is also a barrier to infection with CAR-interacting, Ad5-based vectors. The finding that the Ad5/35 receptor, CD46, is trapped in tight junctions is reminiscent of the situation with the Ad5 receptor, CAR, which is known to be an integral tight junction protein (16). We found that CAR levels were not significantly different between epithelial and mesenchymal ovarian cancer cultures (Supplementary Fig. S13A). As seen with Ad5/35.IR-E1A/TRAIL, epithelial cultures could not be infected from the apical side with an Ad5 vector expressing GFP (Supplementary Fig. S13B). We also observed a correlation between resistance to infection by an Ad5-based oncolytic vector (Ad5.IR-E1A/TRAIL) and the epithelial cell phenotype of ovarian cancer in immunofluorescence and flow cytometry studies (Supplementary Fig. S13C and D). On sections of ovc316 xenografts, CAR was found to be colocalized with the tight junction protein claudin 7 (Supplementary Fig. S14) and in vivo application of Ad5 vectors resulted in similarly inefficient tumor cell transduction as described above for Ad5/35 vectors (data not shown).

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Figure 3. Analysis of adenovirus receptors. A, adenovirus infection of R/E and S/M cells. Left, attachment of $^3$H-labeled particles to cells; middle and right, virus uptake and genome replication. The amount of viral genomes in cells was measured 3 and 72 h after infection with adenovirus vectors at a MOI of 100 pfu/cell by quantitative PCR. B, flow cytometry analysis of surface CD46 and $\alpha_v$ integrins on R/E (resistant) and S/M (susceptible) clones. C, confocal microscopy analysis of CD46, $\alpha_v$ integrin, and claudin 7 on cells that were incubated with Cy3-labeled Ad5/35.IR-E1A/TRAIL (4,000 viral particles/cell) on ice for 30 min (Attachment) or incubated with virus for 2 h at 37°C (Internalization). D, infection of cells with Ad5/35.GFP from the apical and basal sides. Clonal cultures were seeded using tissue culture inserts in 48-well plates and virus was added to medium on top or below cells at a MOI of 10 or 100 pfu/cell. GFP expression was analyzed 48 h later by flow cytometry.
Figure 4. Analysis of ovc316 xenografts. A, analysis of tumor sections. Top, expression of E-cadherin (green) and laminin (red); middle, colocalization of CD46 (green) or αv integrins (green) with the tight junction protein claudin 7 (red); bottom, viral hexon and E-cadherin expression at day 8 post-injection of 2 × 10⁹ pfu of Ad5/35.IR-E1A/TRAIL. In vivo GFP expression after intratumoral (i.t.) injection of Ad5/35.GFP. In vivo β-gal expression after intravenous (i.v.) injection of Ad5/35-β-gal. B, flow cytometry of cell suspensions and cultured ovc316 cells at passages 1 and 20. C, Western blot for key members of pathways that regulate tight junction reorganization and EMT. D, effect of inhibitors [exoenzyme C3 from Clostridium botulinum (Cl. bot.; inhibitor of Rho A, B, and C GTPases), H-1152 (Rho kinase inhibitor), Clostridium difficile (Cl. dif.) toxin B (inhibitor for Rho, Rac, and Cdc42), and wortmannin (inhibitor of phosphoinositide 3-kinase)] on viability of Ad5/35.IR-E1A/TRAIL-infected R/E and S/M cells. Cell viability was measured at day 4 after infection at a MOI of 100 pfu/cell. ***, P < 0.001; **, P < 0.01; *, P < 0.05, compared with infected, mock-treated cells.
Analysis of pathways that are involved in maintaining the epithelial phenotype of ovarian cancer cells. To understand the regulation of tight and adherens junction pathways in ovarian cancer cells, we studied the presence of key members of these pathways in clonal R/E and S/M cultures as well as in xenograft tumors and cultures derived from them at passages 1 and 20 by Western blotting. As expected, we found high levels of E-cadherin in R/E cells and tumors, whereas, with passaging of primary cells, the level of E-cadherin decreased (Fig. 4C). In agreement with the immunofluorescence data (Fig. 1D), N-p120 is expressed at higher levels in S/M clones, and its expression increases with differentiation of E/M hybrid cells into mesenchymal cells during passaging of ovc316 cultures. Differences in N-p120 levels indicate a role of Rho-GTPases in conferring resistance to Ad5/35 infection. Along this line, we found different RhoA levels in R/E and S/M clones. Our Western blot analyses further corroborated that R/E clones...
are closer to tumor cells in vivo and represent a more adequate model for attempts to overcome resistance than populations of primary ovarian cancer cells. Interestingly, expression of ROCK, the downstream effector kinase of Rho, was almost undetectable in R/E cells. Like N-p120, active ROCK is reported to contribute to EMT and the invasive phenotype of epithelial cancers (17, 18), highlighting the importance of mesenchymal features for the susceptibility to viral infection and oncolysis. For focal adhesion kinase, a difference between xenograft tumor and in vitro cultures was observed.

Figure 6. Analysis of infectivity of ovarian cancer cells by different adenovirus serotypes. A, immunofluorescence analyses on R/E cells. Top left, expression of heparan sulfate proteoglycans (green) and claudin 7 (red) on R/E cells; bottom left, Cy3-Ad3 and Cy3-Ad35 attachment in R/E cells (right). Right, effect of Ad3, Ad5, Ad7, Ad11, Ad14, and Ad35 infection (MOI 100 pfu/cell) on cell morphology and E-cadherin (green) expression in R/E cells. Viral replication was visualized by staining for hexon (red). Analysis was done at day 4 post-injection. B, flow cytometry analyses of adenovirus-infected cells (same conditions as in B). C and D, in vivo transduction of wtAd3 and wtAd35. A total of $2 \times 10^7$ pfu of Ad3 and Ad35 was intratumorally injected into subcutaneous ovc316 tumors. C, transduction was quantified by quantitative reverse transcription-PCR for hexon mRNA using pan-serotype hexon primers that can detect both Ad3 and Ad35 hexon mRNA (ref. 23; n = 5). D, ovc316 tumor volume after mock injection or intratumoral injection of $2 \times 10^7$ pfu of wtAd3 or wtAd35 (n = 5).
To validate the role of these pathways in maintenance of epithelial morphology and to potentially manipulate these pathways, we used a series of inhibitors. Treatment of S/M and R/E-EMT cells with Rac/Cdc42/RhoA, rho (A/B/C), or ROCK inhibitors increased their resistance to killing by Ad5/35-IR-E1A/TRAIL. (Fig. 4D). This is most likely due to inhibition of rho family GTPases that are involved in formation of tight adherens junctions and that are also required for efficient integrin-mediated adenovirus internalization and/or intracellular trafficking (19). Surprisingly, unlike GFP expression after infection with Ad5/35-GFP, basal infection of R/E cells with Ad5/35-IR-E1A/TRAIL had no enhancing effect on cell killing compared with apical application of viral particles. This indicates that viral replication and spread is affected in R/E cells even after successful infection. Inhibitors of Rho-GTPases did not increase expression of tight or adherens junction proteins or change the localization of adenovirus receptors in S/M cells. This is in line with our conclusion that defects in regulation of Rho-GTPases represent another independent mechanism that inhibits oncolysis in resistant cells in addition to up-regulated tight and adherence junctions and receptor trapping that prevents adenovirus infection.

**Ad5/35 vectors transduce nonepithelial tumors in vivo.** If our hypothesis that the epithelial phenotype of cancer cells prevents infection by Ad5/35 vectors is correct, tumors derived from nonepithelial cells should be more susceptible to Ad5/35 infection. To test this, we employed a more clinically relevant metastasis model with human cancer cell lines derived from either epithelial tumors [HT-29 (colon cancer) and SAOS (epithelial sarcoma)] or nonepithelial tumors (SKHeP1 (liver endothelial cancer) and HeLa (cervical adenocarcinoma); Fig. 5]. Mice with preestablished liver metastases received a tail vein injection of Ad5/35-GFP or Ad35-injected tumors at day 3 post-injection and continued to increase by day 11, indicating viral replication (Fig. 6C). We also found that a single intratumoral injection of 2 × 10⁹ pfu of wtAd3 significantly delayed tumor growth, whereas Ad35 injection had no therapeutic effect (Fig. 6D). It was impossible to conduct longer studies because mice became moribund after day 10, which we attributed to low-level replication of wild-type virus in normal tissue. At necropsy, we found enlarged livers and spleens, and histologic analysis revealed signs of hepatitis. More detailed therapy studies require therefore the generation of conditionally replicating Ad3 vectors depending on the serotype used for infection were corroborated by flow cytometry studies (Fig. 6B). Mean E-cadherin fluorescence on R/E cells infected with Ad3, Ad7, Ad14, and Ad11 was ~1 order of magnitude less than in Ad5-, Ad35-, and Ad5/35-infected cells. Finally, after intratumoral injection into subcutaneous ovc316 tumors, we found markedly more hexon-positive cells in Ad35-injected tumors than in Ad35-injected tumors (Supplementary Fig. S17). Hexon staining was found in both claudin 7-positive and claudin 7-negative cells, indicating that both epithelial and nonepithelial cancer cell subsets were transduced. The latter was also confirmed by flow cytometry for E-cadherin and GFP (data not shown). In an attempt to quantify in vivo transduction, we measured hexon mRNA levels in total RNA isolated from transduced tumors by quantitative reverse transcription-PCR. Hexon mRNA levels were ~10-fold higher in Ad3-injected tumors compared with Ad35-injected tumors at day 3 post-injection and continued to increase by day 11, indicating viral replication (Fig. 6C). We also found that a single intratumoral injection of 2 × 10⁹ pfu of wtAd3 significantly delayed tumor growth, whereas Ad35 injection had no therapeutic effect (Fig. 6D). It was impossible to conduct longer studies because mice became moribund after day 10, which we attributed to low-level replication of wild-type virus in normal tissue. At necropsy, we found enlarged livers and spleens, and histologic analysis revealed signs of hepatitis. More detailed therapy studies require therefore the generation of conditionally replicating Ad3, Ad7, Ad11, or Ad14 vectors. This task would first require basic studies on DNA replication of these serotypes, which would go beyond the scope of the present article.

**Discussion**

We discovered that the epithelial phenotype of ovarian cancer represents a barrier to oncolysis by oncolytic adenoviruses targeted to CAR, CD46, or αv integrins. Specifically, we showed that these adenovirus receptors were trapped in tight junctions and not accessible to virus binding. Xenograft tumors contained almost exclusively cells in an epithelial or E/M hybrid stage based on their surface markers. Ovarian cancer cells in situ were therefore resistant to infection by CAR- and CD46-targeting adenoviruses. Cells that adapted to tissue culture were in an E/M stage; however, in contrast to E/M cells in vivo, cultured cells already acquired mesenchymal markers such as intracellular N-p120 (Fig. 4C) in a process reminiscent of EMT. Over passing, E/M cells further differentiated into mesenchymal cells, a cell type that is not hypothesized that these adenoviruses were better candidates for achieving infection of R/E cells than Ad5 and Ad35. In support of this, we found that heparan sulfate proteoglycans expressed on E/R cells were not trapped in tight junctions (Fig. 6A, top left). Cy3-labeled Ad3 particles efficiently bound to R/E cells, whereas cell-associated Cy3-Ad35 signals were detectable on only sparse R/E cells (Fig. 6A, bottom left). Importantly, incubation of R/E cells with wild-type adenovirus viruses mediated removal of E-cadherin from the cell surface for Ad3, Ad7, Ad11, and Ad14 but not for the CAR-interacting Ad5 and the CD46-interacting Ad35 (Fig. 6A, right) and Ad5/35 viruses. This study also shows that Ad3, Ad7, Ad11, and Ad14 are able to kill R/E cells, which is reflected in plaque-like foci within the cell monolayer, with hexon-expressing cells along the periphery of the lysis plaques. We also found a clear grouping of adenoviruses in cytolysis assays (Supplementary Fig. S16). Ad3, Ad7, Ad11, and Ad14 were significantly more efficient in lysing R/E cells than Ad5 and Ad35. Changes of membrane E-cadherin in R/E cells depending on the serotype used for infection were corroborated by flow cytometry studies (Fig. 6B). Mean E-cadherin fluorescence on R/E cells infected with Ad3, Ad7, Ad14, and Ad11 was ~1 order of magnitude less than in Ad5-, Ad35-, and Ad5/35-infected cells. Finally, after intratumoral injection into subcutaneous ovc316 tumors, we found markedly more hexon-positive cells in Ad35-injected tumors than in Ad35-injected tumors (Supplementary Fig. S17). Hexon staining was found in both claudin 7-positive and claudin 7-negative cells, indicating that both epithelial and nonepithelial cancer cell subsets were transduced. The latter was also confirmed by flow cytometry for E-cadherin and GFP (data not shown). In an attempt to quantify in vivo transduction, we measured hexon mRNA levels in total RNA isolated from transduced tumors by quantitative reverse transcription-PCR. Hexon mRNA levels were ~10-fold higher in Ad3-injected tumors compared with Ad35-injected tumors at day 3 post-injection and continued to increase by day 11, indicating viral replication (Fig. 6C). We also found that a single intratumoral injection of 2 × 10⁹ pfu of wtAd3 significantly delayed tumor growth, whereas Ad35 injection had no therapeutic effect (Fig. 6D). It was impossible to conduct longer studies because mice became moribund after day 10, which we attributed to low-level replication of wild-type virus in normal tissue. At necropsy, we found enlarged livers and spleens, and histologic analysis revealed signs of hepatitis. More detailed therapy studies require therefore the generation of conditionally replicating Ad3, Ad7, Ad11, or Ad14 vectors. This task would first require basic studies on DNA replication of these serotypes, which would go beyond the scope of the present article.

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present in the tumor in situ. Cells undergoing EMT and mesenchymal cells were susceptible to infection by Ad5 and Ad5/35 vectors. Our findings imply that the cell biology of populations of primary ovarian cancer cell cultures and cancer cells in situ is different and that population cell cultures have only limited value for studying resistance to adenovirus infection. Importantly, clonal R/E cultures were not able to undergo EMT and retained the epithelial phenotype reminiscent of the tumor in situ. The inability of R/E cells to undergo EMT and to maintain the epithelial phenotype seems to be directly linked to the absence and/or inactivity of ROCK in these cells. This speculation is supported by a recent study, showing that Rho-Dia1 signaling stabilized adherens junctions (21). Notably, using tumor cell lysis as the endpoint for resistance studies, expression array studies revealed significantly altered expression for 983 genes, only 33 of which were involved in tight and adherens junction pathways. This indicates that mechanisms other than up-regulated tight and adherens junctions and adenovirus receptor trapping are involved in conferring resistance to viral oncolysis. Although the absence/inactivity of ROCK, and potentially other defects in the regulation of Rho-GTPases, are predictive and functional properties of human bone marrow mesenchymal stem cells. J Cell Physiol 1999;181:67–73.


Epithelial Phenotype Confers Resistance of Ovarian Cancer Cells to Oncolytic Adenoviruses
