Transcriptional Blocks Limit Adenoviral Replication in Primary Ovarian Tumor

Meredith A. Preuss, John T. Lam, Minghui Wang, Charles A. Leath III, Manjula Kataram, Parameshwar J. Mahasreshti, Ronald D. Alvarez, and David T. Curiel

1 Division of Human Gene Therapy and The Gene Therapy Center and 2 Division of Gynecology Oncology, Department of Obstetrics and Gynecology, University of Alabama at Birmingham, Birmingham, Alabama

ABSTRACT

Purpose: Despite the success of conditionally replicating adenoviruses in tumor models, clinical success has been limited when they are used as a single modality agent. Overcoming the disparity in efficacy between in vivo animal models and human use is a key hurdle for better conditionally replicating adenovirus therapy in humans. We endeavored to identify biological blocks to adenoviral infection and replication in tumor cells.

Experimental Design: We hypothesized that the differences in adenoviral replication between ovarian cancer cell lines and patient tumor samples are the result of a block in viral RNA transcription. To test this hypothesis, established ovarian cancer cell lines and purified patient ovarian cancer cells were infected with wild-type adenovirus. RNA for early adenoviral genes E1A and E1B as well as the late transcripts for fiber and hexon were measured using real-time PCR.

Results: Established ovarian cancer cell lines treated with wild-type virus had a lower E1A:E1B ratio than the patient samples. Additionally, the levels of fiber and hexon relative to E1A were also decreased in the patient samples compared with the established cell lines. These findings were consistent with an early- to late-phase block in the adenovirus replication cycle.

Conclusions: These data suggest that the biology of abortive infection in the patient samples may be linked to a defect in the production of early and late viral transcripts.

Identification of factors leading to abortive infection will be crucial to understanding the low viral replication in patient samples.

INTRODUCTION

Conditionally replicating adenoviruses (CRAds) represent a novel therapeutic modality for cancer therapy that has been rapidly translated into the human clinical context (1, 2). However, despite promising data generated in preclinical models, human clinical trials have demonstrated only limited efficacy when CRAds have been used as single modality agents. On this basis, efforts have been made to improve CRAd efficacy by addressing the key biological factors predicating their utility. In this regard, CRAd agents must infect tumor cells efficiently and replicate to generate new progeny virions, which must lateralize to sustain effective amplification.

We have previously noted a significant disparity in expression of the primary adenovirus receptor coxsackie-adenovirus receptor (CAR) in primary tumor cells compared with their cell line counterparts (3, 4). We hypothesized that this CAR deficiency could render tumor cells resistant to adenovirus infection, thus confounding overall CRAd antitumor efficacy. To this end, we have modified adenoviral tropism via genetic capsid modifications such that CAR-independent infection was achieved. Of note, CRAd agents containing such altered tropism exhibited significant augmentations in antitumor efficacy on the basis of the enhanced infectivity of the parent adenovirus (5–11). These efficacy gains suggest that further gains may be achieved by addressing other biological determinants such as replication efficiency.

Considering the key steps of the CRAd replicative cycle, we hypothesize that transcriptional blocks in efficient adenoviral replication, downstream of the initial infection, could also contribute to limited CRAd efficacy. To further address this point, we tested whether the immortalized cell lines used to study CRAd efficacy provided a spurious index of adenoviral replicative capacity analogous to the cell line overestimation of infection susceptibility, as noted above.

Herein, we have evaluated the replicative biology of adenovirus in human ovarian cancer primary tumor samples as compared with immortalized human ovarian cancer cell lines. Our findings indicate the existence of clear blocks to efficient adenoviral replication in primary ovarian tumor cells. This finding provides further insight into the basis of the limited efficacy of CRAd agents noted in the human clinical context. Furthermore, strategies to circumvent tumor cell blocks to adenoviral replication must be identified as a means to realize the full therapeutic potential of CRAd agents.

MATERIALS AND METHODS

Cell Culture. Four established ovarian cancer cell lines were used for these studies. SKOV3.ip1 and OV-4 ovarian
cancer cell lines were obtained from Drs. Janet Price and Judy Wolf (University of Texas M. D. Anderson Cancer Center, Houston, TX). The HEY ovarian cancer cell line was obtained from Timothy J. Eberlein (Harvard Medical School, Boston, MA). PA-1 cells were purchased from American Type Culture Collection (Manassas, VA). Three of the ovarian cancer cell lines (SKOV3.ip1, OV-4, and PA-1) were maintained in RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 25 μg/ml streptomycin. HEY cells were maintained in RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 25 μg/ml streptomycin. All cells were maintained in a humidified atmosphere containing 5% CO2 at 37°C.

Human primary ovarian tumor samples were obtained with institutional review board approval at the time of their initial debulking procedure from patients with pathologically confirmed ovarian adenocarcinoma. The cells were purified from malignant ascites samples using a previously described method for purification of these cells (12). Briefly, most erythrocytes were removed with lysis buffer, and tumor cells were then incubated with CC49 monoclonal mouse antibody (Biological Resources Branch, National Cancer Center Institute, Rockville, MD). Pan Mouse IgG Dynabeads (Dynal AS, Oslo, Norway) coated with antimonos IgG antibodies were then incubated with the cells. Tumor cells were isolated from the benign cells using a magnetic particle concentrator (Dynal AS).

Viruses. Wild-type adenovirus type 5 was obtained from American Type Culture Collection. As a replication-deficient control, we used Adnull virus. This virus was constructed as per the standard AdEASY system procedure (Qiogene, Carlsbad, CA) using homologous recombination with pShuttle (containing no insert) and the pAdEASY-plasmid. The virus was validated by PCR using E1-specific primers to show the presence of E1 in the wild-type virus and a lack of E1 in the Adnull virus.

Human embryonic kidney 293 cells were used to propagate viruses. 293 cells were obtained from American Type Culture Collection. These cells were grown in DMEM:Ham’s F-12 (1:1, v/v) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 25 μg/ml streptomycin. Cells were maintained in a humidified atmosphere containing 5% CO2 at 37°C.

Viral particles were purified on CsCl gradients by standard methods, and the concentration of viral particles was determined by measuring absorbance at 260 nm using a conversion factor of 1.1 x 10¹² viral particles/absorbance unit. For viral infections, viruses were incubated with ovarian cancer cells in the presence of only 2% fetal bovine serum in culture media.

Detection of Virions in Cell Culture Media. All cells were grown in low adherence dishes (Corning ultra-low attachment plates) to facilitate the development of spheroids. Cells were infected at a multiplicity of infection of 1 viral particle/cell in the indicated media with only 2% serum at the time of infection. After 1 h, cells were supplemented with culture media to bring the serum concentration back to 10% for the remainder of the incubation period.

Cell culture media were harvested at days 1, 2, 4, and 6. One μl of fresh cell culture media was analyzed by real-time PCR for fiber transcript. TaqMan primers and probes were designed by Primer Express 1.0 software and synthesized by Applied Biosystems (Foster City, CA). The sequences to amplify adenovirus fiber transcript were as follows: fiber forward, 5’-TGATGTGTGAGCTACAGCCATA-3’; fiber reverse, 5’-GATTTGTGTTGGTGCAATTGGTG-3’; and fiber probe, 5’-ACCAAATCAAGCCCATCTCCTGCATAATG-3’. With optimized concentration of primers and probe, the components of real-time PCR mixture were designed to result in a master mix with a final volume of 9 μl/reaction containing 1× TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems), 100 nM forward primer, 100 nM reverse primer, 100 nM probe, and 0.025% BSA. For the assay, a known amount of adenovirus template DNA (10⁶, 10⁵, 10⁴, and 10² copies/μl) was amplified to generate a standard curve for quantification of the adenovirus copy numbers of unknown samples. One μl of total RNA sample was added to 9 μl of PCR mixture in each reaction capillary. A “no template” control received 1 μl of water. All capillaries were then sealed and centrifuged using 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 as follows: 30 min at 48°C; 10 min at 95°C; and 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were analyzed with LightCycler software version 3.

Detection of Viral Transcripts in Cells. Cells were cultured and infected as described above. At the indicated time points, cell culture media were removed, and the remaining cells were collected by scraping into PBS. This solution was centrifuged, and supernatant was removed. RNA obtained from the pelleted cells was stabilized immediately using RNAlater reagent (Qiagen) and stored at –80°C until extraction. RNA was isolated using the standard protocol provided by Qiagen for RNAeasy isolation. Contaminating DNA was removed by DNase digestion (Qiagen).

TaqMan primers and probes were designed by Primer Express 1.0 software and synthesized by Applied Biosystems. The sequences to amplify adenovirus genes were as follows: E1A forward, 5’-AACCGATTGTGGCGGTAGGTC-3’; E1A reverse, 5’-CTCGTTAAGCACTCTCCTGATA-3’; E1A probe, 5’-ACACGGCTTGAGGCGCCA-3’; E1B forward, 5’-TTTTGGCAGTCTGCTTCT-3’; E1B reverse, 5’-GCGGACGGGAGACAAATAG-3’; E1B probe, 5’-AGCCATTCCCTCGGCTCACAACACCCT-3’. All real-time PCR reactions were performed under the same conditions as described above.

Western Blots. Media containing cell spheroids were collected and pelleted in a conical tube. Plates were scraped with cold PBS and collected into a minimal volume of PBS. After centrifugation, the supernatant was removed, and the cell pellet was stored at –20°C until analysis. Before analysis, samples were dissolved in cell lysis buffer containing SDS to disrupt the cell membrane. Using PAGE, 10–30 μg (as required) of total cell protein were separated on a 12.5% gel. After transfer to polyvinylidene difluoride (90 min at 100 V), the membrane was then blocked with a 5% milk/TBTS solution [TBTS, 0.02 M...
RESULTS

CRAd agents kill target tumor cells as a consequence of their replicative cycle, a process termed oncolysis. Amplification of this effect is critically linked to effective production of progeny virions that infect adjacent tumor cells. We thus initially evaluated the ability of tumor target cells to produce new virions as a consequence of adenoviral infection. We used wild-type adenovirus in these experiments as a CRAd surrogate and as a means to study the basic biology of the adenovirus life cycle in tumor immortalized cell lines versus primary tumors. For this analysis, human immortalized ovarian cancer cell lines and primary tumor isolates were infected with wild-type adenovirus or a replication-defective control virus at a multiplicity of infection of 1 viral particle/cell. Cell culture media were evaluated at various times after infection for the presence of progeny virions.

In these studies, the immortalized cell lines exhibited a time-dependent increase in the number of progeny virions present in the cell culture media, noted initially by day 4 postinfection and increasing exponentially through the end of the analysis at day 6 (Fig. 1). Of note, in the human primary tumor material derived from three different patients, a time-dependent increase in progeny virions was also noted, albeit to a much lesser extent. Virions present in the cell culture media only increased up to 4-fold in the primary patient tumors in 6 days as compared with the 60–810-fold increase observed in the immortalized cell lines. It thus appeared that there were substantial differences in the ability of immortalized cell lines and primary tumors to support productive adenoviral infections.

These findings are consistent with a primary resistance to adenoviral infection and/or a defect in the adenoviral replication, after infection, in the target cells. In this regard, we have noted such an adenovirus-resistant phenotype in primary tumor cells with respect to adenoviral vector transduction. Therefore, we sought to normalize for differences in infection susceptibility between cell lines and primary tumor while analyzing the specific steps in the adenoviral replicative cycle. To this end, we designed PCR primers to the adenoviral proteins (designed for the transcripts) for E1A, E1B, fiber, and hexon to evaluate adenoviral transcription in infected target cells. Early transcription events (E1A:E1B ratio) and later steps in the replicative cycle (hexon:E1A and fiber:E1A) were studied. Analysis of the ratios instead of the absolute levels of each transcript would provide a direct index of the ability of the adenovirus replicative cycle to progress in infected target cells, independent of the efficiency of target cell infection.

To analyze the early and late events in adenoviral replication using this index, the different cell lines were infected with a multiplicity of infection of 1 viral particle/cell of wild-type adenovirus or a replication-defective control virus. E1A transcripts were measured as well as E1B transcripts (Fig. 2A), represented as the ratio of E1A:E1B. In the primary patient samples, there was a 7-fold decrease in the ratio of early transcripts. Although not statistically significant, this may be indicative of a block early in the replication of the adenovirus that was independent of the infectivity. It was also apparent from these data that the established cell lines contained more E1A transcript and also retained the ability to effectively progress the infection of the wild-type adenovirus. These data suggest an early replicative block that may limit the progression of adenovirus infection in the patient samples, independent of infection susceptibility. The high levels of E1B transcript may be a result of flawed E1B being produced, thus limiting the progression of the early infection.

To determine whether this early block was the unique transcriptional defect in the ovarian cancer patient samples, we next sought to examine the late genes expressed after infection. PCR primers were designed for fiber and hexon as indicators of transcripts expressed late in infection. At 48 h after wild-type virus infection, these late mRNA transcripts were measured and compared with E1A. As seen in Fig. 2B, the ratio of fiber transcripts:E1A was 9-fold higher in the immortalized cell lines versus the primary patient samples. These data indicate that the primary patient samples are unable to support the transcription
of fiber as effectively as the immortalized cell lines. To determine whether this effect was related to fiber alone or to other late transcripts, we also analyzed the transcription of hexon at 48 h after infection compared with E1A. Interestingly, the ratio of hexon:E1A is 1.8-fold higher in the primary patient samples compared with the immortalized cell lines (although the difference is not statistically different, \( P = 0.27 \)). Thus, there is no significant defect in the transcription of hexon in the primary patient samples.

Because replicative defects were present in the early and late phases of the adenovirus replicative cycle in the primary patient samples, it was expected that there would be a similar decrease in the translation of the corresponding proteins. To examine whether there was a defect in the translation of these critical proteins, we used Western blot analysis to determine the levels of each protein in the primary tumor samples and immortalized cell lines (Fig. 3A). E1A proteins were detectable in all samples, however, E1A was highest in patient 41 and SKOV3.ip1 cells. This corresponded to the data shown in Fig. 1; patient 41 produced the most progeny of the patient samples, and SKOV3.ip1 cells also gave a productive infection. PA-1 cells had little E1A protein but had the most productive infection compared with all cell types. This is an interesting observation that warrants further investigation into the posttranslational modification of these key regulators of early infection. Although fiber protein was detected in all samples, its level was profoundly higher in the two immortalized cell lines that also gave the most productive infection (PA-1 and SKOV3.ip1). Similar to fiber, hexon was also present in all samples, but it was expressed at the highest level in the two immortalized cell lines that were able to best support productive infection (PA-1 and SKOV3.ip1). In total, high levels of fiber and hexon protein correlated with productive infection in the immortalized cell lines.

To assess the relationship of fiber and hexon production independent of infectivity, we required a similar index to that used for the mRNA transcripts as seen in Fig. 2. Thus, we chose to also calculate the ratio of each protein level with E1A. Densitometric ratios were calculated in triplicate. Representative blots are shown in Fig. 3A, whereas Fig. 3B and C, shows the densitometric analysis. Similar to what was observed in the measurement of mRNA, fiber:E1A protein ratios were decreased in the primary patient samples compared with the cell lines. The levels of translated fiber decreased, possibly due to the decreased transcription of fiber mRNA. Finally, the hexon: E1A protein ratio was high in all samples, with patient 38 leading in production of hexon relative to E1A. Patient 38 had low levels of E1A, increasing the value of the ratio. Again, PA-1 and SKOV3.ip1 gave the most productive infection and had the highest hexon protein levels of the immortalized cells.

**DISCUSSION**

The oncolytic potency of CRAds is critically linked to effective adenoviral infection and replication. First, there must be efficient infection in target cells. To this end, we have shown gains in infectivity via CAR-independent infection to overcome the hurdle that most tumor cells are deficient with respect to the level of the CAR receptor. These gains provide a logical framework for addressing other biological factors related to CRAd potency, such as efficacy of replication, as studied here. Furthermore, elucidation of distinct mechanisms will allow us to develop a strategy to circumvent other barriers and improve the potency of CRAds. In the present study, we endeavored to define the replicative blocks that may be limiting the full potential of oncolytic CRAds.

We showed that immortalized cell lines support wild-type adenoviral replication to a much greater degree than primary patient tumors. Because fiber and hexon are both late proteins involved in the assembly of virions, it was expected that high levels of these proteins would correlate with the cell lines in which the adenovirus had the most productive infection. However, increased levels of E1A did not correlate with the produc-
tivity of infection. In fact, previous studies have indicated that factors present in the infected cells can influence this process and thus affect the ability of the virus to replicate effectively in the host cell. For example, serine-rich proteins within the cell have been shown to suppress viral infection by blocking the obligate splicing of adenoviral pre-mRNA (15). Furthermore, the decrease in adenoviral replication may be due to ineffective or absent cleavage of polyadenylated RNA (16). In both of these cases, specific factors expressed by the cells may influence the outcome of productive infection. Thus, the presence of defects at multiple steps of the replicative cycle in the patient samples is consistent with the overall concept that the decreased replication of adenovirus results from an aggregation of defects.

Similar defects in virus replication were observed in cross-species abortive infection. This phenomenon is illustrated by the lack of viral replication in certain species host cell types such as a human adenovirus in murine host cells. In the literature, a prominent model for studying this phenomenon includes the infection of monkey cells with human adenovirus type 2. In this model, transcription of fiber mRNA was decreased 5–20-fold, and the translation of fiber was decreased to 100-1000-fold. It has been hypothesized over the last two decades that this is a 2-fold defect that is manifested not only in transcription but also as an impediment in translation of the protein due to incomplete splicing or incomplete maturation of the fiber mRNA. This host cell restriction on translation was overcome by cotransfection with SV40 (17–20). SV40 DNA provided unknown factors necessary to allow the progression of the adenovirus type 2 infection. Circumvention of another abortive cross-species infection has been demonstrated using human adenovirus serotype 12, which is known not to replicate in hamster cells. However, coinfection with human adenovirus serotype 2 or 5 led to the production of replication-competent progeny virions (21). Finally, baby hamster kidney cells are nonpermissive for adenovirus serotype 12. This abortive infection was bypassed by overexpressing the adenovirus serotype 5 E1 region in cell culture. In this case, E1 region overexpression was sufficient to circumvent transcriptional blocks in this model (22). These studies indicate that expression of viral genes or coinfection with other viruses may provide the unknown factors required to allow the progression of an abortive infection.

The limited replication of wild-type adenovirus in patient primary samples parallels this model. In this study, we defined the basic biology of abortive replication in the primary tumor cells as having the following polyfactorial contributors: (a) decreased expression of early genes; (b) decreased expression of late genes; and (c) decreased translation of the late gene fiber. These factors are strikingly similar to those seen in cross-species abortive infection, which warrants the study of coinfection as a means of circumventing these blocks. It is noteworthy that selected molecules will circumvent replicative blocks in cross-species abortive infections. Similar strategies in primary tumors might also be suggested to induce significant gains in the oncolytic potency of CRAVs.
REFERENCES


Transcriptional Blocks Limit Adenoviral Replication in Primary Ovarian Tumor

Meredith A. Preuss, John T. Lam, Minghui Wang, et al.


Updated version  Access the most recent version of this article at: [http://clincancerres.aacrjournals.org/content/10/9/3189](http://clincancerres.aacrjournals.org/content/10/9/3189)

Cited articles  This article cites 22 articles, 10 of which you can access for free at: [http://clincancerres.aacrjournals.org/content/10/9/3189.full.html#ref-list-1](http://clincancerres.aacrjournals.org/content/10/9/3189.full.html#ref-list-1)

Citing articles  This article has been cited by 1 HighWire-hosted articles. Access the articles at: [http://clincancerres.aacrjournals.org/content/10/9/3189.full.html#related-urls](http://clincancerres.aacrjournals.org/content/10/9/3189.full.html#related-urls)

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.