Virus-Associated RNA I–Deleted Adenovirus, a Potential Oncolytic Agent Targeting EBV-Associated Tumors

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Abstract

Given the growing number of tumor types recognizably associated with EBV infection, it is critically important that therapeutic strategies are developed to treat such tumors. Replication-selective oncolytic adenoviruses represent a promising new platform for anticancer therapy. Virus-associated I (VAI) RNAs of adenoviruses are required for efficient translation of viral mRNAs. When the VAI gene is deleted, adenovirus replication is impeded in most cells (including HEK 293 cells). EBV-encoded small RNA1 is uniformly expressed in most EBV-associated human tumors and can functionally substitute for the VAI RNAs of adenovirus. It enables replication to proceed through complementation of VAI-deletion mutants. We hypothesized that VAI-deleted adenovirus would selectively replicate in EBV-positive tumor cells due to the presence of EBV-encoded small RNA1 with no (or poor) replication in normal or EBV-negative tumor cells. In this report, we show that high levels of replication occurred in the VAI-deleted mutant in the EBV-positive tumor cells compared with low (or negligible) levels in EBV-negative and normal human primary cells. Correspondingly, high toxicity levels were observed in EBV-positive tumor cells but not in EBV-negative tumor or normal human primary cells. In vivo, VAI-deleted adenovirus showed superior antitumoral efficacy to wild-type adenovirus in EBV-positive tumor xenografts, with lower hepatotoxicity than wild-type adenovirus. Our data suggest that VAI-deleted adenovirus is a promising replication-selective oncolytic virus with targeting specificity for EBV-associated tumors. (Cancer Res 2005; 65(4): 1523-31)

Introduction

EBV, a ubiquitous human herpesvirus found latently expressed in 90% of the human population, is the causative agent of infectious mononucleosis and posttransplant immunoproliferative disorders in immunocompromised patients. It is closely associated with the development of a variety of malignant diseases, including endemic Burkitt's lymphoma, B-cell lymphoma of immunocompromised patients, nasopharyngeal carcinoma, Hodgkin's disease, T-cell lymphoma, natural killer cell leukemia/lymphoma, smooth-muscle tumors, and gastric cancer (1, 2). More recent reports have linked EBV with conventional epithelial cancers of other sites, including breast (3–5), lung (6–9), prostate (7), liver (10), colon (7), and also with lymphoepithelioma-like carcinoma of the esophagus (11). Given the ever-growing number of tumor types associated with EBV infection, therapeutic strategies to treat EBV-associated tumors may be considered a high priority in oncology.

Replication-selective, oncolytic viruses provide a new platform to treat cancer. Promising clinical trial data with mutant adenoviruses have shown both their antitumor potency and safety (12, 13). Two main approaches are currently being used to engineer adenoviruses with tumor-selective replication. The first strategy limits the expression of the E1A gene to tumor tissues through the use of tumor- and/or tissue-specific promoters; consequently, E1A-induced S phase entry and activation of viral and cellular genes will only occur in tumor tissues. A second strategy optimizes tumor selectivity by deletion of viral genes that are critical for efficient replication in normal cells but expendable in tumor cells (14). The most commonly described oncolytic viruses explored over the past 10 years have been mutant adenoviruses where at least 25 different ones have been described for use in cancer treatments to date (15).

Several studies have shown similarities in the function of adenovirus and EBV genes (16–20). Based on the similarities described, we believe that a window of opportunity exists in the design of replication-selective adenovirus for EBV-associated tumors. The adenovirus genome encodes two RNA polymerase III-directed, ~160-nucleotide-long RNAs, the so-called virus-associated RNA I and RNA II. These accumulate to high levels during the late stages of viral infection (21–23). VAI RNA is obligatory for efficient translation of viral and cellular mRNAs (24, 25). It binds to and blocks the activation of cellular double-stranded RNA-dependent serine/threonine protein kinase (PKR), which phosphorylates protein translation initiation factor eIF-2α, enabling protein synthesis to proceed at normal levels (26–29). A mutant that fails to produce VAI RNA (dl331) grows more poorly than its parent in human 293 cells (25).

EBV also expresses two low-molecular-weight RNAs, designated EBV-encoded RNAs (EBER) 1 and 2, transcribed by RNA polymerase III (30). Although there is no striking nucleotide sequence homology between virus-associated RNAs and EBERs, the RNAs are similar in size, genomic organization, and degree of secondary structure; they have similar functions (19, 20, 31). In particular, the two small RNAs encoded by EBV can efficiently complement the VAI RNA–mediated translational defect in adenovirus-infected cells, as shown by constructing an Ad5 substitution mutant in which the two virus-associated RNA genes have been deleted and replaced by an EBV DNA segment encoding the two EBERs (32, 33). Given the uniform expression...
of EBERs in human EBV-associated tumors, generally at high levels, and the similar functions between VAI RNA and EBER1, we hypothesized that VAI RNA–defective adenovirus could be complemented by EBER1 in EBV-positive tumors, thus restricting virus-induced cell killing and replication to EBV-positive tumors. In the present report, we tested this hypothesis. We show that VAI-deleted adenovirus is a potential therapeutic agent that can specifically target EBV-associated tumors.

**Materials and Methods**

**Cell Lines and Cell Culture.** Human Burkitt's lymphoma (Raji), epidermoid carcinoma of larynx (Hep-2), lung cancer (A549), and embryonic kidney (HEK; a subclone named JH-293) cell lines were all obtained from the Cancer Research UK Central Cell Service (Clare Hall, Middlesex, United Kingdom). The Jijoye cell line was obtained from American Type Culture Collection (Manassas, VA) and the human nasopharyngeal carcinoma line C666-1 from Prof. Dorly P. Huang (The Chinese University of Hong Kong, Hong Kong, China). The presence of EBV has been consistently shown in C666-1 (34). Raji, Jijoye, Hep-2, and C666 cells were maintained in RPMI containing 10% fetal bovine serum, 2% L-glutamine, and antibiotics. JH-293 and A549 cells were maintained in RPMI containing 10% fetal bovine serum, and antibiotics. The human gastric cancer cell lines AGS and AGS-EBV were a generous gift from Prof-T Lindsey Hutt-Fletcher (Louisiana State University, Shreveport, LA). The AGS-EBV cell line was obtained by G418 selection of AGS cells that were infected with a recombinant Akata virus in which a neomycin resistance cassette had been inserted into the nonessential BDLF3 open reading frame. Both the AGS and AGS-EBV cell lines were maintained in Ham's F12 medium, supplemented with 10% fetal bovine serum, and antibiotics, and G418 at 500 µg/mL (AGS-EBV cells only). Normal human bronchial epithelial cells (NHBE) and normal prostate epithelial cells (PrEC) were obtained from Clonetech Corp. (San Diego, CA) and were propagated as recommended by the manufacturer. All cells were maintained at 37°C in a humidified atmosphere containing 10% CO2.

**Viruses.** VAI-deleted adenovirus d331 (25), wild-type Ad5 and E3B-deleted adenoviral mutant d309 (35) were gifts from Prof. Bayar Thimmappaya (Northwestern University, Chicago, IL) and Dr. Thomas Shenk (Princeton University, Princeton, NJ). The replication-defective Ad5 mutant d312 was inactivated by psoralen-UV, as previously described, to serve as a nonreplicating particle control for in vitro cell survival and in vivo efficacy studies. Ad5, d309, and d312 adenoviruses were amplified in the human embryonic kidney cell line HEK 293; d331 was generated in A549 cells. All adenoviruses were purified and titered as previously described (36).

**Viral Infection and Replication Assay.** Cells were seeded in six-well plates (3 × 10^4 cells/well, according to their growth properties) and infected with viruses at 100 particles per cell when 70% to 80% confluence had been reached. For all human tumor cells, viral infections were done in standard growth medium without serum. Primary human cells were infected in predefined growth medium, with no alterations to growth factor components. Infections were done in a low volume of medium for 2 hours at 37°C/10% CO2. Medium was then replaced either with fresh medium containing 2% fetal bovine serum for tumor cells or with growth factors for primary human cells. Experiments were repeated under each condition in triplicate. Cells and media were collected at 48 and 72 hours postinfection. The collected samples were freeze-thawed thrice, and replicating virus was determined by the limiting dilution assay as described (36) with the use of A549 and 293-T cells (293 cells transformed with SV40 T antigen) as indicator cells. A549 supports VA-deleted adenovirus replication to an equivalent level to wild-type adenovirus (37), and SV40 T antigen (in 293-T) could rescue the translational defect resulting from infection with VA-deleted adenovirus (38).

**In vitro Cell Survival Assay.** For cell survival assays, 1 × 10^4 cells (1 × 10^5 cells of Hep-2) were seeded in each well of 96-well plates in 100 µL medium and infected 24 hours later with serial dilutions of different viruses. Cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium assay (MTS, Promega, Madison, WI) 6 days postinfection, and the EC50 values of different adenoviruses obtained as previously described (39). For NHBE and PrEC cells, 100 µL fresh medium was added to each well well 3 days postinfection. All assays were done at least thrice with each concentration of virus in sextuplicate.

**Detection of EBER1 Expression by Reverse Transcription-PCR.** Total RNA was extracted from different cells, using a RNA extraction kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's instructions. To synthesize cDNA, 1 µg of total RNA was treated with 10 units of DNase I (Roche, Lewes, United Kingdom) at 37°C for 30 minutes, as described (40). After purification, DNase I-treated RNA was reverse transcribed by Superscript II reverse transcriptase (Invitrogen, Renfrew, United Kingdom) with 50 pmol pd(N6), random hexamer primers (Amersham Biosciences, Chalfont St. Giles, United Kingdom) in 20 µL. For PCR amplification, 1 µL cDNA was added to 49 µL PCR reaction mixture [75 mM Tris-HCl (pH 8.8), 20 mM (NH4)2SO4, 0.01% (vol/vol) Tween 20, 1.5 mM MgCl2, 0.16 mM (each) deoxynucleoside triphosphate, and 30 pmol of each primer] containing 1.5 units of TaqDNA polymerase (Agrgene, Epom, United Kingdom). To analyze expression of EBER1, the primer pairs used were 5'-AGAGCACCAGCTGCTCCATGAA-3' (forward) and 5'-CCCCATCAGTTGTGTCCGAGAACAC-3' (reverse). After 30 cycles of amplification, PCR products were separated by electrophoresis on 1% agarose gels containing ethidium bromide. The human β-actin gene was used as an internal control.

**Western Blotting.** Control and infected cells were lysed at indicated times with lysis buffer [20 mMol/L Tris-HCl (pH 7.6), 400 mMol/L NaCl, 50 mMol/L KCl, 5 mMol/L β-mercaptoethanol, 1% Triton X-100, 20% glycerol, 100 µg/mL phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, and 10 µg/mL aprotonin]. Protein lysates were quantitated by the bicinchoninic acid assay (Sigma, Poole, United Kingdom). Proteins were separated by reducing SDS-10% polyacrylamide gel electrophoresis, electrodtransferred onto nitrocellulose membranes and probed with a mouse anti-total PKR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or a rabbit anti-phospho-PKR (Biosource, Camarillo, CA), or rabbit anti-Elf-2α (p52) phosphospecific antibody (Biosource), or mouse anti-human proliferating cell nuclear antigen antibody (Research Monoclonal Antibody Service, Cancer Research UK, London, United Kingdom). Immunocomplexes were detected by incubation with a horseradish peroxidase–conjugated secondary antibody (DAKO, Glostrup, Denmark) and visualized by enhanced chemiluminescence reagent (Amersham Biosciences).

**In vivo Efficacy, Replication, and Gene Expression.** Two approaches were used to evaluate the antitumor efficacy of VA-deleted adenovirus and wild-type Ad5 in vivo as described below. All animal experiments were approved by the Cancer Research UK Animal and Safety Committee and the United Kingdom Home Office.

In the first protocol, cultured C666-1 cells were infected with vehicle buffer, or psoralen-UV-inactivated d312, wild-type Ad5, and d331, for 2 hours at 1,000 particles per cell. Fourteen hours postinfection, 10% preinfected C666-1 cells mixed with 90% uninfected cells, and 1.5 × 10^5 mixed cells, in 200 µL of PBS, were injected s.c. into the flanks of 6 BALB/c nu/nu mice (4-6 weeks old) for each group. Tumor growth, as measured by volume, was monitored twice weekly.

In the second protocol, 2 × 10^5 C666-1 cells were suspended in 200 µL normal saline and injected s.c. into the flanks of athymic (nu/nu) mice (4-6 weeks old) and allowed to grow. Volume was estimated twice weekly using the formula: volume = (length × width)^3/3.142/6. Once tumors reached the size range of 33.5 to 117 mm^3 for 30 days postinfection, and the EC50 values of different adenoviruses obtained as previously described (39). For NHBE and PrEC cells, 100 µL fresh medium was added to each well well 3 days postinfection. All assays were done at least thrice with each concentration of virus in sextuplicate.
studies, tumors were injected once only, on day 1. Tumors were harvested at stated time points after injection for analysis of viral gene expression, replication, and general histopathology as described (15). Tumor size and animal survival were monitored. Animals were sacrificed when tumor reached the size limited by Home Office regulations or after 3 months. Survival analysis was done according to the method of Kaplan-Meier.

**Viral Hepatotoxicity.** Murine lung adenocarcinoma CMT-64 (5 × 10⁶) cells, were injected s.c. into both flanks of immunocompetent C57/BL6 mice (obtained from Cancer Research UK Biological Resources). The animals were randomized into four groups (n = 9 per group) and treated with PBS, PUVd331, wild-type Ad5, and dl331. I.v. injection of viruses (1 × 10⁶ particles in a volume of 100 μl) via the tail vein was initiated once the tumor size reached 40 to 80 mm³. At 24, 48, and 72 hours postinjection, mice were sacrificed (three mice per time point), liver samples were collected and processed for general histopathology, and viral protein expression was analyzed as described (15).

**Statistical Analysis.** Unpaired t test or one-way ANOVA were used for all in vitro studies and in vivo toxicity studies. Kaplan-Meier curves were compared using the log-rank test.

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**Table 1. Human cells evaluated and EBER1 expression**

<table>
<thead>
<tr>
<th>Cell name</th>
<th>Derived tissues</th>
<th>EBER1 status</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHBE</td>
<td>Normal human bronchial epithelial cells</td>
<td>–</td>
</tr>
<tr>
<td>PrEC</td>
<td>Normal human prostate epithelial cells</td>
<td>–</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonal kidney cells</td>
<td>–</td>
</tr>
<tr>
<td>A549</td>
<td>Human lung cancer cells</td>
<td>–</td>
</tr>
<tr>
<td>Panc-1</td>
<td>Human pancreatic cancer cells</td>
<td>–</td>
</tr>
<tr>
<td>Hep-2</td>
<td>Human epidermoid carcinoma of larynx</td>
<td>–</td>
</tr>
<tr>
<td>AGS</td>
<td>Human stomach cancer cells</td>
<td>–</td>
</tr>
<tr>
<td>AGS-EBV</td>
<td>AGS-transfected with EBV</td>
<td>+</td>
</tr>
<tr>
<td>Jioyoe</td>
<td>African Burkitt’s lymphoma cells</td>
<td>+</td>
</tr>
<tr>
<td>Raji</td>
<td>African Burkitt’s lymphoma cells</td>
<td>+</td>
</tr>
<tr>
<td>C666-1</td>
<td>Human nasopharyngeal carcinoma</td>
<td>+</td>
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**Results**

**EBER1 Expression in Normal Human Primary and Transformed Cells.** To determine the selectivity of replication of VAI-deleted adenovirus targeting EBV-associated tumors and cell killing, a panel of human cell lines including normal primary and EBV-negative and EBV-positive tumor cell lines was used, as listed in Table 1. Given that EBV resides in 90% of the human population, the expression pattern of EBER1 in all cells was predetermined by semiquantitative reverse transcription-PCR before testing our complementation hypothesis. Raji, Jioyoe, AGS-EBV, and C666-1 expressed varying levels of EBER1 RNA whereas other cells were negative (Fig. 1; Table 1). Among the four EBV-positive cell lines, the highest EBER1 RNA expression levels were observed in the C666-1 and Raji cells, whereas slightly lower levels were detected in the AGS-EBV cells previously stably transfected with the EBV genome.

**Selectivity of EBER1-Positive and EBER1-Negative Cells to Oncolysis by VAI-Deleted Adenovirus.** EC₅₀ values were determined in each cell to compare the cell killing potencies of VAI-deleted and wild-type adenovirus in normal and tumor cells with different EBER1 status (Table 1). The ratios of EC₅₀ value for dl331 and wild-type Ad were compared and correlated to EBER1 status in each cell line (Fig. 2A). In the panel of human cells examined, the dl331 mutant showed a cell killing potency that was equivalent or superior to wild-type adenovirus in the EBV-positive AGS-EBV, Raji, Jioyoe, and C666-1 cells. In contrast, the dl331 mutant was markedly attenuated in the EBV-negative human cells tested both in tumor cell lines and normal primary human cells. Surprisingly, Panc-1 cells, a human ras mutant pancreatic cancer cell line previously reported to be susceptible to dl331 (41), showed a very low level of cytopathic effect to dl331 in the present study. These results show that the VAI-deleted adenovirus could selectively kill EBER1-positive cell lines, whereas they have little effect on EBV-negative and normal cells. In addition, the VAI-deleted adenovirus was as potent as wild-type adenovirus in the EBER1-positive tumor cell lines.

**Viral Replication of VAI-Deleted Adenovirus in Normal Primary Human, EBV-Negative and EBV-Positive Tumor Cells.** To determine whether dl331-induced selective cell killing was indicative of replication induction in EBV-positive cells, replication of mutant and wild-type adenovirus was compared in individual cell lines. The EBV-positive (AGS-EBV, Jioyoe, Raji, and C666-1), EBV-negative (JH-293, Panc-1, Hep-2, and AGS-ATCC) and normal human primary (NHBE and PrEC) cells were infected with VAI-deleted and wild-type adenoviruses, and cells were harvested at 48 or 72 hours. The VAI-deleted mutant replicated to levels of 20% to 73% that observed for wild-type Ad5 in all four
EBV-positive tumor cell lines (Fig. 2B). The highest level of replication of dl331 was observed in C666-1 cells, which also express the highest level of EBER1 RNA among these cells. Viral production by VAI-deleted adenovirus in EBV-negative cells was lower than in EBV-positive cells. To our surprise, viral production by VAI-deleted adenovirus in normal primary cells was 4,000 times less than wild-type adenovirus in NHBE, and 500 to 1,000 times less in PrEC cells, respectively. Of note, among EBV-negative human tumor cell lines, only Panc-1 showed a relative level of replication, that is ~20% that of wild-type Ad5 at 72 hours.

Because in each cell line, viral infection, replication, lysis, and spread are dependent on their intrinsic properties, it may not be reasonable to compare two tumor cell lines derived from different sources. Therefore, data from matched cell lines, such as AGS and AGS-EBV, could be more meaningful. The viral replication ratio of dl331/Ad5 in AGS-EBV at 48 hours proved to be 16 times ($P < 0.01$; Fig. 2B).

These results show that VAI-deleted adenovirus can selectively replicate in EBV-positive tumor cells with no significant replication being observed in normal primary cells.
Changes in PKR and eIF-2α Activation in Response to Viral Infection. VAI RNA regulates mRNA translation and controls viral replication through the PKR/eIF-2α pathway. EBER1 has a similar function (19, 42, 43). To test whether the selectivity of the VAI mutant for EBV-positive cells occurs through a blocked PKR/eIF-2α pathway, total PKR, phosphorylated-PKR, and phosphorylated-eIF-2α were analyzed by immunoblotting in EBV-positive cells (C666-1) and EBV-negative cells (Hep-2 and Panc-1) at 24 hours postinfection with wild-type Ad5, dl309, and dl331 (Fig. 3). Viral infection resulted in increased expression levels of total PKR in all these cell lines and there was no difference in the level of total PKR between viruses (Fig. 3, 1). In normal cells, PKR is active through a mechanism involving autophosphorylation in response to viral infection. Changes in phosphorylated PKR levels were therefore determined in the three cells following infection with different adenoviruses (Fig. 3, 2). As expected, dl331 infection of EBV-negative cells showed a significant elevation of phosphorylated PKR compared with wild-type adenovirus infection. However, EBV-positive C666-1 cells still showed elevated levels of phosphorylated PKR following dl331 treatment, whereas infection with wild-type Ad5 and dl309 mutant blocked PKR phosphorylation (Fig. 3, 2). We further investigated phosphorylation of the substrate of PKR, eIF-2α, using a rabbit anti-eIF-2α[pS52] phosphospecific antibody. As observed in Fig. 3, 3, infection with dl331 resulted in a minor increase in the level of phosphorylated eIF-2α in comparison with wild-type adenovirus infection in EBV-positive cells; however, dl331 produced a significantly elevated level of phosphorylated eIF-2α compared with wild-type adenovirus in EBV-negative cells. These results indicate that EBER 1 may complement the regulation of eIF-2α by a route not exclusively dependent on PKR. In addition, no differences in phosphorylated PKR and phosphorylated eIF-2α levels were observed in normal human primary cells following wild-type or VAI-deleted adenovirus infection (data not shown) although the cells showed a significant difference in viral replication with low or no replication occurring with VAI mutant.

Antitumoral Efficacy of the VAI-Deleted Adenovirus In vivo Is Equivalent or Superior to that of Wild-type Adenovirus In EBV-Positive Tumors. To evaluate whether dl331 could inhibit tumor growth in vivo, preinfected C666-1 cells were implanted s.c. in BALB/C nu/nu mice as described in Materials and Methods. Tumor formation and growth were monitored over time. Tumors formed in all PBS-treated control animals and in 83.3% (5 of 6) of animals that received inactivated, nonreplicating control adenovirus at 27 days postimplantation. No tumor formed in any of the animals that received 10% of wild-type Ad5- or dl331-preinfected cells up to 205 days postimplantation with the mixed tumor cells. Statistical analysis confirmed significant differences between both of wild-type Ad5- and dl331-treated groups and each control group (P < 0.01).

The antitumor efficacy of dl331 was also confirmed in preestablished s.c. C666-1 tumors treated by intratumoral virus administration as described in Materials and Methods. Tumor growth and survival rate over time were monitored (Fig. 4A and B). The dl331 mutant significantly inhibited tumor growth compared with the two control groups, and was superior to wild-type Ad5 (Fig. 4A). Significantly prolonged survival was also observed in the dl331-treated animals compared with Ad5-treated animals (Fig. 4B). The dl331 and Ad5 treated groups had 75% and 50%, respectively, animals alive after 75 days compared with controls with no animals surviving after 50 days.

To confirm cell death and active viral replication in the tumor mass, H&E and immunohistochemical staining with antiviral protein E1A and hexon antibodies were done on cross sections taken on day 5 after single viral injections (Fig. 4C). H&E staining confirmed the killing of tumor cells from live virus-treated tumors, with dl331 resulting in more severe cytopathic effects than wild-type adenovirus (Fig. 4C, 1). Equivalent levels of viral proteins E1A and hexon were observed in EBV-positive tumors after single treatments with dl331 and wild-type adenovirus (Fig. 4C, 2 and 3), demonstrating that viral replication had occurred within the tumor cells.

VAI-Deleted Mutant Results in Reduced Hepatotoxicity In vivo. Intratumoral treatment with replication-selective oncolytic adenovirus is well tolerated in both mice and humans (44). However, systemic administration might have higher toxicity risks in organs such as liver. The systemic delivery of replication-competent adenoviruses, even when modified for tumor-selective replication, has previously been shown to cause dose-limiting toxicity in mice, associated with liver necrosis resulting from direct exposure to adenoviral coat proteins, viral early gene expression, viral replication, and/or de novo late gene expression (45). The host immune response may also play a role in mediating this dose-limiting toxicity. To explore this topic, the acute hepatotoxicity of dl331 and wild-type adenoviruses in the recently developed CMT-64 immunocompetent mouse tumor model (36) was investigated, as described in Materials and Methods. Histopathologic observations showed that cytopathic effects of hepatocytes following dl331 treatment decreased with time, whereas those of wild-type adenovirus were consistently higher at three time points. Viral late gene (hexon) expression and cytopathic effects in the liver after dl331 treatment were significantly lower than that found with wild-type adenovirus.

**Figure 3.** PKR and eIF-2α alterations in EBV-positive and EBV-negative tumor cell lines after infection with dl331 and wild-type adenovirus. Cells were infected with Ad5, dl309, or dl331 at 100 particles per cell as described in Materials and Methods. Twenty-four hours later, proteins were extracted, separated by gel-electrophoresis, and immunoblotted for total PKR, phosphorylated PKR (P-PKR), phosphorylated eIF2α (P-eIF2α), and proliferating cell nuclear antigen (PCNA). Lane 1, uninfected-cells; lane 2, wild-type Ad5-infected cells; lane 3, dl309-infected cells; lane 4, dl331-infected cells. Data are from duplicate experiments.
Figure 4. Antitumoral efficacy of VAI deletion mutant (d/331) in established C666-1 (EBV-positive) tumors in vivo. C666-1 human nasopharyngeal carcinoma xenografts implanted s.c. in C57BL/6 nu/nu mice as described in Materials and Methods. PBS or viruses (1 × 10¹⁵ particles) were injected intratumorally on 5 consecutive days. Single injections were done for the biological time-points study. A, tumor growth curves; animals treated with d/331 (n = 7) and wild-type Ad5 (n = 6) showed significant delays in tumor growth compared with the control groups injected with PBS (n = 7) or the inactivated particles control group (n = 7; P < 0.01). B, animal survival curves. d/331 and wild-type Ad5 treatment produced significantly higher survival rates than the control group (P < 0.001); the survival rate of d/331-treated mice was superior to Ad5. C, representative histologic changes and viral protein expression in C666-1 xenografts on day 5 after treatment with different adenoviruses, as indicated. H&E staining (original magnification, ×200) shows that Ad5 and d/331 produce significant cytopathic effects in tumor cells (i.e., cell death, eosinophilic cytoplasm, enlarged nuclei, and inclusion bodies). Control tumors show no significant change. Immunohistochemical analysis of such sections (original magnification, ×200) shows that the remaining tumor cells stain positively for viral proteins E1A and hexon (brown), whereas the PBS-treated tumors show no immunoreactivity; the PUVd/312-treated tumors show only weak nuclear staining for the hexon coat protein and no E1A staining.
after 48 hours: There was no difference of E1A expression between wild-type and VAI-deleted adenoviruses infection. The viral gene hexon expression was consistent with the cytopathic effect alteration. Figure 5 represents examples of histopathology and adenovirus hexon protein staining in liver after viral treatment.

Discussion

The presence and expression of the EBV genome undoubtedly contributes to the development of many, if not all, tumors associated with this virus, opening up an unique opportunity to develop novel anticancer treatments directed against viral, rather than cellular, targets. A number of therapies have been proposed to target EBV-associated tumors based on expression of viral genes, as recently reviewed (46). Some examples are those inhibiting the expression of EBV-encoded oncogenes or induction of EBV episome loss. Others involve the use of EBV-dependent promoters and replication origins to express toxic genes, preferentially within EBV-positive tumors, the deliberate induction of the lytic EBV replication in tumor cells, or various immune therapies targeting EBV viral proteins. In the present study, we exploited the fact that EBERs are uniformly expressed in most human EBV-associated tumors, engineering a complementary mutation in a replication-competent adenovirus to produce a selective oncolytic viral therapy for these tumors. Indeed, a VAI-deleted mutant, dl331, showed selectivity for EBV-positive cells with the therapeutic index being especially advantageous for these cells as opposed to normal primary human cells. The data presented here show that this mutant could be used to treat EBV-associated human tumors.

VAI RNA–PKR–eIF-2α has been recognized as a major pathway in host defense against viral infection and replication. Based on common functions exhibited by adenovirus VAI RNA and Ras for inactivating PKR, it was recently reported that a VAI mutant adenovirus could be used selectively to treat tumors with an activated ras oncogene (41). In the present study, VAI-deleted adenovirus showed selective cell killing and replication in EBV-positive tumor cells but not significant in a tumor cell line (Panc-1) with activated Ras pathway. Our results show that in the EBV-positive tumor-derived cell line C666-1, dl331 induced a higher level of phosphorylated-PKR but a minor increase level of phosphorylated-eIF-2α when compared with wild-type adenovirus. These findings suggest that EBER may act through another pathway independent of PKR to affect host protein translation.

It is known that the phosphorylation of eIF-2α is mediated by at least three different protein kinases: the IFN-inducible dsRNA-activated kinase PKR, the erythroid cell-specific, heme-regulated kinase HCR (HRI) and the nutrient-regulated protein kinase GCN2 (47). A recent report (48) showed that EBER-1 could enhance protein synthesis by a PKR-independent mechanism, strongly supporting our data. In addition, SV40 antigen can rescue the translational defect in monkey and human cells that result from infection of VAI-deleted adenovirus. However, the large T antigen of SV40 does not prevent the activation of PKR and the complementation appears to occur at a step downstream of PKR activation, maybe by dephosphorylation of eIF-2α (38), and EBER1 may act through a similar pathway. In the EBV-negative tumor cell lines, Hep-2 and Panc-1, dl331 induced higher levels of phosphorylated-PKR and phosphorylated-eIF-2α than wild-type adenovirus although Panc-1 has a mutant ras oncogene that could be expected to inactivate PKR. These results are consistent with the poor cell killing and viral replication observed in response to dl331 in these cells. However, 72 hours after infection with dl331, viral replication in Panc-1 cells approached 20% of that of wild-type adenovirus, comparable with the observation in some EBV-positive cells. This suggests that partial complementation occurred in the cells but there was very low cytotoxicity with dl331 in Panc-1 cells compared with
adenovirus; however, no differences were observed in the level of good selectivity of viral replication compared with wild-type molecular mechanism for this.

deaminase. Further investigation will be required to explore the antagonizing the RNA-editing activity of the ADAP adenosine deaminase. Lei et al. (49) reported that adenovirus VAI RNA may affect viral and the 2-5 (A) synthetases, which also affect viral replication (20, 31). Lei these cells. Indeed, VAI RNA of adenovirus, like EBER-1, can activate RNA apparently has other functions that control viral replication in vitro showed superior cell killing in vivo and antitumoral efficacy in vivo compared with wild-type adenovirus, in EBV-positive cells, although the absolute level of viral replication of dl331 was lower than wild-type adenovirus. Our data indicate that EBV-positive C666-1 cells still have a relatively high level of phosphorylated PKR after dl331 infection. PKR has been reported to be indispensable for cell apoptosis by apoptotic stimulators such as E2F-1 and tumor necrosis factor-α (50, 51), and activation of PKR can induce the expression of Fas and trigger apoptosis through the Fas-associated death domain protein/caspase-8 death signaling pathway (52, 53). Thus, it is reasonable to postulate that higher PKR levels in dl331-infected cells may promote adenovirus-induced cell death and this would explain the lower level of replication in these cells. In addition, an increasing production of IFN-β in the absence of VAI RNA may also explain why dl331 showed superior cell killing and antitumoral efficacy compared with wild-type adenovirus. VAI RNA also has similar functions to E3L of vaccinia virus. Deletion of the E3L has been shown to activate IFN regulatory factor 3 (IRF3) and increase IFN-β production in cells infected by vaccinia virus lacking this gene (54). Of note, dl331 was derived from dl327, which has a large deletion in the adenoviral E3 region (25). This deletion may be another factor that affects the antitumoral efficacy because we have found that E3B deletion of adenovirus can enhance potency compared with wild-type adenovirus in vitro and in vivo in nude mice but not in immunocompetent mouse tumor models (15).

Although dl331 has superior antitumoral efficacy compared with wild-type adenoviruses in vivo, the proportion of cases (2 of 7) showing complete regression of tumor after viral treatment alone was still low. It would therefore be desirable to develop other strategies that maintain selectivity but enhance the efficacy of the adenovirus. Based on our findings (15, 55), deletion of the E1B 19K gene but retention of E3B seems promising in this regard. In addition, it is conceivable that combination of adenovirus and conventional approach, such as chemotherapy, radiotherapy, and immunotherapy, will improve antitumor efficacy.

Replication-selective, oncolytic adenoviruses have been evaluated extensively in epithelial tumors but relatively little in lymphoid malignancies mainly owing to the perceived resistance of the latter to adenovirus infection. However, in the present study, we found that this resistance is not absolute and that, whereas most lines are refractory, adenovirus can infect some B-cell lymphoma lines, such as the Burkitt’s lymphoma derived lines Raji and Jijoye, resulting in cytotoxicity to these cells. This is consistent with observations described elsewhere using primary chronic lymphocytic leukemia and B-cell lymphoma lines (56). Taken together, our findings suggest that oncolytic adenovirus is also a potential agent to treat at least a proportion of lymphomas. We found that the infectivity of Burkitt’s lymphoma lines is associated with the expression of adenovirus receptors, such as cossackievirus adenovirus receptor and heparan sulfate glycosaminoglycans, but that genetic alterations also play an important role in the cytotoxicity of adenoviruses in lymphoma cells (57); the mechanism behind the latter finding is not understood at present.

In conclusion, our data suggest that a VAI-deleted adenovirus can induce replication and cell lysis selectively in EBV-positive tumor cells. The replication-selective, oncolytic adenovirus showed efficient tumor killing in vitro and in vivo for EBV-positive tumor cells, and lower hepatotoxicity than wild-type adenovirus. It represents a potential therapeutic agent for targeting human EBV-associated tumors, an increasingly important group of malignant diseases.

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