Adeno-Associated Virus Enhances Wild-Type and Oncolytic Adenovirus Spread

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Abstract
The contamination of adenovirus (Ad) stocks with adeno-associated viruses (AAV) is usually unnoticed, and it has been associated with lower Ad yields upon large-scale production. During Ad propagation, AAV contamination needs to be detected routinely by polymerase chain reaction without symptomatic suspicion. In this study, we describe that the coinfection of either Ad wild type 5 or oncolytic Ad with AAV results in a large-plaque phenotype associated with an accelerated release of Ad from coinfected cells. This accelerated release was accompanied with the expected decrease in Ad yields in two out of three cell lines tested. Despite this lower Ad yield, coinfection with AAV accelerated cell death and enhanced the cytotoxicity mediated by Ad propagation. Intratumoral coinjection of Ad and AAV in two xenograft tumor models improved antitumor activity and mouse survival. Therefore, we conclude that accidental or intentional AAV coinfection has important implications for Ad-mediated virotherapy.

Introduction
Oncolytic viruses offer a unique opportunity to treat cancer because selective replication in tumor cells multiplies the amount of cells that become infected, a major limitation in cancer gene therapy. Among different viruses, adenovirus (Ad) has been extensively modified to achieve tumor selective replication (Alemany, 2007). However, despite promising preclinical results, limited efficacy in over 20 clinical trials suggests that Ad oncolytic potency needs to be improved (Russell et al., 2012). A potent oncolytic Ad should efficiently release its progeny to facilitate rapid spread through the tumor. Despite that Ad release is key for efficient oncolysis (Gros, 2010), it is slow, as it occurs late in the virus life cycle when the adenovirus death protein (ADP) has accumulated (Tollefson et al., 1996b). Moreover, Ad release is inefficient, as less than 20% of the total virus produced is released within 2 days postinfection. Mathematical models that combine parameters of Ad replication rate, tumor growth rate, and immune response point to virus release as a key factor for efficacy (Wein et al., 2003). Ads with deleted E1B19K, truncated i-leader protein, or overexpressing ADP show faster progeny release and higher oncolytic activity (Sauthoff et al., 2000; Ramachandra et al., 2001; Doronin et al., 2003; Puig-Saus et al., 2012), highlighting Ad release as a key parameter for intratumor spread and antitumor efficacy.

Adeno-associated viruses (AAVs) were first described in 1965 as nonautonomous viruses contaminating Ad stocks. AAVs are nonpathogenic defective paroviruses. The name “AAV” reflects the inability to efficiently replicate in the absence of a helper virus, such as Ad, human papilomavirus or herpes simplex virus. Several AAV serotypes have been described (Gao et al., 2004; Schmidt et al., 2006) with different tissue tropism (Grimm et al., 2003; Muller et al., 2006). Studies on AAV-Ad interaction frequently involve AAV2 and Ad wild type 2 (Ad 2) or Ad wild type 5 (Ad5). AAV2-Ad coinfection leads to lower levels of Ad gene and protein expression in an AAV dose-dependent manner, affecting certain Ad transcripts and proteins more than others (Timpe et al., 2006). AAV contamination has been associated with lower Ad yields (burst size) (Hoggan et al., 1966; Carter et al., 1979). However, the effect of AAV contamination on Ad cytoxicity is unclear, with a lower cytotoxicity described in HeLa cells (Jing et al., 2001) and an enhanced cytolysis and apoptosis in A549 cells (Timpe et al., 2007).

Considering the previously described effects of AAV on Ad yield and cytotoxicity and the current interest on virotherapy with Ads, we studied the effect of AAV on Ad

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HUMAN GENE THERAPY METHODS 24:372–380 (December 2013) © Mary Ann Liebert, Inc.
DOI: 10.1089/hgtb.2013.124
release, cytotoxicity, and oncolytic potency. Ad formed large plaques when combined with either AAV2 or AAV6, a phenotype caused by a faster and more efficient Ad release from infected cells. Of note, AAV also enhanced cell death and improved therapeutic activity of an oncolytic Ad, opening the possibility to use AAV in Ad-mediated virotherapy.

**Materials and Methods**

**Cell lines and viruses**

HEK293 and A549 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). NP9 cells were established in our laboratory (Villanueva et al., 1998). All cell lines were routinely tested for mycoplasma and were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS), penicillin (50 mg/ml), and streptomycin (50 mg/ml) at 37°C and 5% CO2. Human Ad5 was obtained from ATCC. Oncolytic Ad ICOVIR15 is described elsewhere (Rojas et al., 2010). AAV serotype 2 was generated by transfection of HEK293 monolayers with psub201 and a plasmid supplying helper genes as previously described (Casper et al., 2005). AAV serotype 6 was generated by pAAV6 (kindly provided by Dr. David W. Russell, Department of Medicine, University of Washington, Seattle, WA) and helper plasmid transfection of HEK293. AAV6 was purified over iodixanol gradient as described (Zolotukhin et al., 1999). AAVs were titrated by quantitative polymerase chain reaction.

**Cell-plaque assay**

A549 monolayers were infected with Ad5 or ICOVIR15 at a multiplicity of infection (MOI) of 50, or coinfected with either Ad5 or ICOVIR15 (50 MOI) plus either AAV2 or AAV6 at 1,000 viral particles (vp)/cell. Four hours postinfection, cells were washed with phosphate buffered saline (PBS) and trypsinized. About 20 μl of each cell suspension was diluted 1/5 in trypsin blue, and cells were counted in a Neubauer chamber. Fifty cells of each infection condition were added on top of near-confluent A549 monolayers seeded in six-well plates. After 4 hr, the medium was removed and cells were covered with a 1:1 mix of DMEM–5% FBS:1% agarose. Once agarose solidified, DMEM–5% FBS was added. To better visualize plaques, monolayers from the different infection conditions were stained by incubation with 0.5 mg/ml thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO) at 37°C and 5% CO2 for 4 hr.

**Virus release and production assays**

HEK293, A549, and NP9 monolayers seeded in 24-well plates were infected in triplicate at an Ad MOI that allowed 100% infectivity (5 for HEK293; 50 for A549 and NP9 cells) and at different vp/cell of AAV6 depending on the cell line (100 for HEK293; 100, 1,000, 10,000, and 100,000 for A549; and 1,000 for NP9). Four hours after infection, cultures were washed thrice with PBS and incubated in a fresh virus-free medium. At the indicated time points after infection (time 0 meaning immediately after addition of the fresh medium), a small fraction of the supernatant (SN) was collected, and the cells and the medium were harvested and frozen-thawed three times to obtain the cell extract (CE). Viral titers were determined by an antithexon staining-based method (Cascollo et al., 2007). A two-tailed Student’s t-test was used to study statistical significance.

**Cytotoxicity assays**

A549 monolayers seeded in six-well plates were mock-infected or infected with ICOVIR15 (50 MOI) alone or combined with AAV6 at 1,000 vp/cell. Four hours postinfection, cells were washed thrice with PBS and incubated with fresh virus-free DMEM–5% FBS at 37°C and 5% CO2. At the indicated time points, samples were collected and analyzed for lactate dehydrogenase (LDH) levels (expressed in IU/ml/liter) in the Biochemistry Service of the Bellvitge Hospital following the protocol established by the International Federation of Clinical Chemistry. The absorbances at 430 and 700 nm were determined for each sample on a Cobas c711 (Roche Diagnostics, Basel, Switzerland) spectrophotometer, calibrated with a c.f.a.s. (Roche Diagnostics, ref. 759350) using reagent LDH 1 Gen. 2 (Roche Diagnostics, ref. 04964560 022). The significance of differences between groups was assessed by a two-tailed Student’s unpaired t-test. For the trypsin blue exclusion assay, SNs were removed at the indicated time points. Cells were trypsinized and combined with SNs. Cell and SN mixture was diluted 1/10 in trypsin blue, and cells were counted in a Neubauer chamber. Cells were counted in groups of 100 (500–600 cells for each time point and condition). Statistical significance between groups was assessed by a two-tailed Student’s unpaired t-test. For cell morphology, unfixed cells were microscopically examined and photographed at the indicated time points postinfection with a Leica DMIL LED light microscope and Leica Application Suite LAS v.2.6 software (Leica, Wetzar, Germany).

**In vitro propagation assays**

Propagation assays were performed by seeding 30,000 A549 or NP9 cells per well in 96-well plates in DMEM–5% FBS. Cells were infected by triplicate with serial dilutions of ICOVIR15 alone, starting with 200 transducing units (TU)/cell, or coinfected with four AAV6 doses, starting with 400, 4,000, 40,000, or 400,000 vp/cell for A549 or NP9 cells. These doses were chosen to match the number of functional AAV and Ad particles considering an Ad dose of 200 MOI and a similar TU/vp ratio for both viruses; thus, we used 4,000 vp of AAV. We used one lower dose and two higher doses in order to demonstrate a dose-dependent effect of AAV on Ad propagation-mediated cytotoxicity. At day 5 postinfection for A549 cells and day 6 postinfection for NP9 cells, plates were washed with PBS and stained for total protein content (bicinchoninic acid assay; Pierce Biotechnology, Rockford, IL). Absorbance was quantified and the TU per cell required to produce 50% of culture growth inhibition (IC50 value) was estimated from dose–response curves by standard nonlinear regression (GraFit; Erithacus Software, Horley, United Kingdom), using an adapted Hill equation. A two-tailed Student’s t-test was used to study the statistical differences between the IC50 values of different groups. For assays using coinfected cells, monolayers of A549 or NP9 cells were infected with ICOVIR15 alone (50 MOI) or combined with 1,000 vp/cell of AAV6. Four hours postinfection, cells were washed once with PBS, trypsinized, and counted in a Neubauer chamber. Serial dilutions of infected cells, starting with...
1,000 A549 cells or 3,000 NP9 cells, were done by triplicate on 96-well plates and 30,000 A549 or NP9 noninfected cells were added, respectively. At day 7 for A549 cells and day 9 for NP9 cells, the same protocol of total protein staining, quantification, and statistical study as above was performed. In this case, IC50 values were calculated as the number of infected cells needed to produce 50% of culture growth inhibition.

In vivo antitumoral efficacy

Animal studies were performed at the IDIBELL animal facility (AAALAC unit 1155) and approved by the IDIBELL’s Ethics Committee for Animal Experimentation. Subcutaneous A549 or NP9 carcinoma tumors were established by injection of 1.5×10⁶ or 3×10⁶ cells, respectively, into the flanks of 6-week-old female Balb/C nu/nu mice (Harlan Laboratories, Venray, B.V. Netherlands). To minimize the number of animals used, each animal was implanted with two tumors, one in each flank. When tumors reached 150 mm³ (experimental day 0; n = 8–10 tumors), mice were randomized and were injected with a single intratumor injection of PBS, 2×10¹⁰ vp of AAV6, 2×10⁹ vp of ICOVIR15, or 2×10¹⁰ vp of AAV6 and 2×10⁹ vp of ICOVIR15 in a volume of 20 µL in PBS. Tumor volume was defined by the equation, V (mm³) = π/6 × W² × L, where W and L are the width and the length of the tumor, respectively. Data are expressed as relative tumor size to the beginning of the therapy. The statistical differences in relative tumor size between treatment groups were assessed by a two-tailed Student’s unpaired t-test. For Kaplan–Meier survival curves, the end point was established at ≥500 mm³. The survival curves from the different treatments were compared. Animals whose tumor size never achieved the threshold were included as right-censored information. A log-rank test was used to determine the statistical significance of the differences in time-to-event.

Results

Ad-AAV coinfection results in a large-plaque phenotype

We performed a cell-plaque assay to compare the plaque phenotypes of cells infected with Ad alone or Ad and AAV. We infected A549 cells with Ad5 or ICOVIR15 (Rojas et al., 2010), each of them either alone or combined with AAV2 or AAV6. Fifty cells from each infection condition were added on top of near-confluent A549 monolayers and were covered with agarose to force the viruses to spread from cell to cell. Plaques derived from cells coinfected with Ad and either AAV2 or AAV6 appeared earlier and had a larger size at any given time point, for both Ad5 (Fig. 1a) and ICOVIR15 (Fig. 1b).
1b). Interestingly, cells coinfected with AAV6 developed faster and larger plaques than AAV2; thus, we focused on AAV6.

**Coinfection with AAV6 enhances ICOVIR15 release but decreases burst size**

ICOVIR15 yield and release in the presence or absence of AAV6 were quantified in three cell lines (Fig. 2). HEK293 (human embryonic kidney) are commonly used to propagate Ads. A549 (lung adenocarcinoma) and NP9 (pancreatic adenocarcinoma) are tumor cell lines commonly used as models in virotherapy. Cells were synchronically infected with ICOVIR15 alone or combined with AAV6. At the indicated time points, SNs and CEs were harvested and Ad was tittered using an antihexon staining-based method (Cascallo et al., 2007). In HEK293 cells (Fig. 2a), AAV6 increased ICOVIR15 release 10-, 15-, and 7-fold at 24, 48, and 72 hr postinfection, respectively (*p* < 0.05), but decreased 1.87- and 2.17-fold ICOVIR15 total production at 48 and 72 hr postinfection, respectively (*p* < 0.05). In A549 cells (Fig. 2b), coinfection with AAV6 increased ICOVIR15 release 87-, 34-, and 2.5-fold at 24, 48, and 72 hr postinfection, respectively (*p* < 0.05), but decreased total production 1.6-, 4.7-, and 6.75-fold at 24, 48, and 72 hr postinfection, respectively (*p* < 0.05). In NP9 cells (Fig. 2c), AAV6 enhanced ICOVIR15 release 13- and 5-fold compared with ICOVIR15 at 48 and 72 hr postinfection, respectively (*p* < 0.05). Total production was not affected in NP9 cells. A similar enhanced Ad release and lower Ad production was observed when infecting A549 cells with Ad5 and AAV6 (Supplementary Fig. S1; Supplementary Data are available online at

![FIG. 2. Ad-AAV coinfection enhances Ad release.](image)
www.liebertonline.com/hgtb). No differences of Ad content in total CE at time point 0 were observed in any cell line (Supplementary Fig. S2).

We extended the study on AAV6 effects on ICOVIR15 release and total production by coinfecting A549 cells with different AAV6 doses. We measured the relative amounts of Ad released (virus released/total virus produced) using four AAV6 doses (100, 1,000, 10,000, and 100,000 vp/cell) to find which dose enhanced ICOVIR15 release more efficiently (Fig. 2d). In all the time points analyzed, the dose of 1,000 vp/cell induced the highest relative Ad release. This dose was also the dose that induced the highest absolute amounts of virus released (Supplementary Fig. S3).

The viability of ICOVIR15-infected cells is reduced by AAV6

To detect morphology changes at early times associated to AAV6 coinfection, A549 cells were infected with ICOVIR15 alone or ICOVIR15 and AAV6. As described (Timpe et al., 2007), coinfected cells showed an apoptotic-like morphology characterized by cell shrinkage and membrane blebbing as soon as 36 hr postinfection (Fig. 3a). Cells infected with ICOVIR15 alone showed minimal signs of cytopathic effect at the same time point (36 hr), but complete cytopathic effect at 72 hr postinfection. To further study this accelerated cell death, we studied plasma membrane integrity and cell lysis.

![Cell morphology at the indicated hours postinfection](a)

![Cell lysis measured by LDH release into the culture medium](b)

![Percentage of viable cells determined](c)

<table>
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<tr>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>ICOVIR15</th>
<th>ICOVIR15/AAV6</th>
<th>ICOVIR15/AAV6</th>
<th>ICOVIR15/AAV6</th>
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<tr>
<td>A549</td>
<td>0.015±0.006</td>
<td>0.014±0.002*</td>
<td>0.014±0.002*</td>
<td>0.006±0.001*</td>
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<tr>
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<td>0.27±0.017*</td>
<td>0.118±0.009*</td>
<td>0.06±0.003*</td>
<td>0.062±0.005*</td>
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<tr>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Cells infected with ICOVIR15</th>
<th>Cells infected with ICOVIR15/AAV6</th>
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<tbody>
<tr>
<td>A549</td>
<td>12.42±1.959</td>
<td>0.961±1.121 *</td>
</tr>
<tr>
<td>NP9</td>
<td>1002.67±188.291</td>
<td>72.12±15.664 *</td>
</tr>
</tbody>
</table>

* p≤0.05 by two-tailed unpaired Student’s t-test compared with mock-infected cells.

![Comparative dose–response curve of ICOVIR15 alone or combined with AAV6](d)

![The IC<sub>50</sub> values (infected cells needed to cause a reduction of 50% in cell culture viability) obtained from comparative dose–response curve of A549 or NP9 cells infected with ICOVIR15 alone or combined with AAV6 at day 7–8 after infection are shown. Three different replicates were quantified for each cell line. Mean±SD are shown. * p≤0.05 by two-tailed unpaired Student’s t-test compared with cells infected with ICOVIR15.](e)
using the same coinfection conditions. Cell lysis, measured by LDH release, showed a marked increase \((p < 0.01)\) in coinfected cells compared with ICOVIR15-alone infected cells at early times (24 and 36 hr postinfection) (Fig. 3b). At later times (48 and 72 hr postinfection), lysis was not significantly different compared with ICOVIR15-infected cells. LDH levels in noninfected cells remained basal during all the experiment. Plasma membrane integrity and cell viability were monitored by trypan blue exclusion (Fig. 3c). Loss of membrane integrity and cell viability of coinfected cells appeared as soon as 24 hr postinfection. A minority of cells were viable 36 hr postcoinfection with ICOVIR15 and AAV6 (9.8%, 7.7%, and 1.7% of cells were viable at 36, 48, and 72 hr postcoinfection, respectively). In contrast, the majority of cells were viable at 36 and 48 hr postinfection with ICOVIR15 (95% and 85%, respectively). Viability was 11-fold higher for ICOVIR15-alone infected cells compared with coinfect ed cells (85.3% vs. 7.7%, \(p < 0.01\)) at 48 hr postinfection. Noninfected cell viability remained near 100% during all the experiment.

**AAV6 enhances the ICOVIR15 propagation-mediated cytotoxicity in vitro**

Cell monolayers were infected with low doses of viruses (achieved by serial dilutions) and incubated for several days to allow multiple rounds of replication. Such propagation-mediated cytotoxicity assays (IC\(_{50}\) assays; IC\(_{50}\) amount of virus needed to reduce cell culture viability by 50%) were performed by infecting preseeded monolayers of A549 or NP9 cells with viruses or by seeding infected cells over them. To prepare virus mixtures, four initial different doses of AAV6 (400, 4,000, 40,000, and 400,000 vp/cell) and one initial dose of ICOVIR15 (200 transducing units [TU]/cell) were used. The results showed that AAV6 enhanced dose dependently the propagation-mediated cytotoxicity of ICOVIR15 (lower IC\(_{50}\) values) in both cell lines (Fig. 3d). Similar results were obtained when seeding monolayers with serial dilutions of cells infected with ICOVIR15 or ICOVIR15 and AAV6. Coinfection strongly enhanced cytotoxicity in both cell lines, decreasing dramatically the IC\(_{50}\) values (Fig. 3e). Comparative dose–response curves from both assays are shown in Supplementary Fig. S4.

**ICOVIR15-AAV6 injection enhances antitumor activity and prolongs mice survival after intratumor administration**

Once we had demonstrated the ICOVIR15-AAV6-enhanced cytotoxicity in vitro, we wanted to determine whether it conferred a therapeutic advantage in vivo. Mice bearing A549 or NP9 tumors were treated intratumorally with PBS, AAV6 \((2 \times 10^{10} \text{ vp/tumor})\), ICOVIR15 \((2 \times 10^{7} \text{ vp/tumor})\), or a mixture of ICOVIR15 and AAV6 at these same doses in a single injection (Fig. 4a). The AAV6 dose was 10-fold higher than the dose of ICOVIR15 to increase tumor cell coinfection probability. In the A549 model, coinfection reduced tumor growth compared with PBS- and AAV6-injected groups from day 14 postinjection (p.i.) \((p < 0.05)\) until day 38 of treatment, when PBS and AAV6 groups had to be sacrificed because of uncontrolled tumor growth. ICOVIR15 reduced tumor growth compared with AAV6 at days 35 and 38 after treatment \((p < 0.05)\). Coinfection reduced 2.6-fold the tumor growth compared with ICOVIR15 injection at the end of the experiment (day 66), although this difference was not statistically significant. In the NP9 model, the combination of both viruses resulted in a marked inhibition of tumor growth. ICOVIR15-AAV6 coinfection decreased tumor growth compared with control groups (PBS and AAV6) from day 20 p.i. \((p < 0.05)\) and compared with ICOVIR15 from day 29 p.i. \((p < 0.05)\). At the end of the study (day 48 after treatment), coinfection inhibited tumor growth 1.9-fold compared with ICOVIR15. Kaplan–Meier analysis (Fig. 4b) showed a survival benefit for the group treated with both viruses compared with controls in A549 and NP9 models \((p < 0.05)\), and compared with ICOVIR15 group in NP9 model \((p < 0.05)\). ICOVIR15 treatment showed a trend to improve the mean survival compared with control groups (PBS and AAV6) in both models although did not achieve statistical significance.

In summary, our results indicate that Ad coinfection with AAV induces a large-plaque phenotype caused by an increase in Ad release, which enhances Ad cytotoxicity in vitro and antitumor efficacy in vivo.

**Discussion**

A clear phenotype associated to AAV contamination of Ad stocks is the lower Ad production yield (Hoggan et al., 1966; Carter et al., 1979; Timpe et al., 2006). As Ad, human papilloma virus (HPV) can also function as an AAV helper, and lower HPV yields have also been observed after AAV coinfection. Intriguingly, it has been described that AAV accelerates the HPV life cycle (Agrawal et al., 2002). Here we describe that the combination of Ad5 or oncolytic Ad ICOVIR15 with either AAV2 or AAV6 results in earlier and larger-plaque phenotype. This phenotype is long-lived (14 days postinfection; not shown), suggesting an improvement in propagation that is sustained during multiple replication rounds. As a large-plaque phenotype correlates with enhanced antitumor activity of oncolytic Ads, we attempted to elucidate if the AAV-induced large-plaque phenotype could be applied to virotherapy. AAV2 has been largely used as a reference serotype for AAV, but we also included AAV6 in our analysis to extend the observations to another AAV serotype. Compared with AAV2, AAV6 produced even faster and larger Ad plaques. AAV6’s high capability for airway epithelia transduction is well documented; however, the infectivity of AAV6 in A549 lung adenocarcinoma cells is not superior to AAV2 (Grimm et al., 2008), suggesting that such tropism for lung is not the cause for the AAV6 larger-plaque phenotype. Hence, we focused on serotype 6 for oncolytic studies.

Ad large-plaque phenotype has been associated to enhanced progeny release (Sauthoff et al., 2000; Dororon et al., 2003; Gros et al., 2008). Accordingly, AAV6 enhanced ICOVIR15 release in all cell lines analyzed. However, the time after infection when we detected the highest difference on Ad release induced by AAV was different for each cell line. NP9 showed a delay in this AAV-induced Ad release that could be associated to a lower permissiveness to Ad replication (lower production yields) than A549 and HEK293 cells. In HEK293 and A549 cells, AAV6 decreased Ad production as expected (Carter et al., 1979; Timpe et al., 2006). Moreover, this phenotype was not restricted to an oncolytic Ad, as AAV6 also enhanced Ad5 release. The amount of Ad
present in the total CE immediately after the coincubation time (time point 0) was not influenced by the presence of AAV (Supplementary Fig. S2), suggesting that the AAV’s effect on Ad is not associated to initial virus entry.

As Ad is affected by AAV in a dose-dependent manner (Timpe et al., 2006), a range of coinfection conditions with AAV6 were used to evaluate the effects on ICOVIR15 release and total production in A549 cells. Compared with other doses, the dose of 1,000 vp/cell resulted in the highest ratio of released virus to total produced virus and, importantly, in the highest absolute amount of virus in the SN with complete virus release after 48 hr postinfection. Hence, looking only at Ad presence in the SN, the enhanced release overcomes the lower production. We did not find a dose-dependent correlation, as lower or higher doses than 1,000 vp/cell had less impact on virus release.

Several strategies to enhance Ad release have been described: ADP overexpression (Doronin et al., 2003), apoptosis induction by deletion of genes such as E1B19K (Sauthoff et al., 2000) or insertion of proapoptotic transgenes (Sauthoff et al., 2002), and bioselection of mutagenized Ads (Subramanian et al., 2006; Gros et al., 2008). ADP overexpression and apoptosis induction accelerate cell death (Sauthoff et al., 2000; Doronin et al., 2003; Subramanian et al., 2006). It has also been observed that AAV induces a characteristic apoptotic cell morphology on Ad-infected cells, with cell shrinkage and membrane blebbing (Timpe et al., 2007). We observed this apoptotic-like morphology (Fig. 3a) as soon as 36 hr after coinfecting with AAV6 at 1,000 vp/cell. This AAV-induced apoptotic-like phenotype correlated to accelerated cell death and cell membrane disruption, which were measured by LDH secretion and trypan blue exclusion. This enhanced cytotoxicity correlates with previous reports of increased cytolysis and apoptosis during the first 3 days after infection of A549 cells with Ad5 and AAV2 (Timpe et al., 2007). However, Jing et al. (2001) saw opposite results in HeLa cells that could be related to the different cells used. Our results in A549 and NP9 cells support those of Timpe et al. 2007 in favor of an AAV-induced cytotoxicity.

The mechanism whereby AAV increases Ad cytotoxicity is not clear. Here we describe an enhanced release with premature cell death and a characteristic apoptotic-like morphology. Previous reports indicate that AAV Rep proteins induce apoptosis (Zhou and Trempe, 1999; Zhou et al., 1999; Schmidt et al., 2000; Timpe et al., 2007) and that AAV Rep protein expression is inhibited at high AAV doses (Carter et al., 1979; Timpe et al., 2006), decreasing theoretically the proapoptotic effects. In the other hand, adenoviral E1B19K and ADP proteins are related with viral spread, apoptosis, and cytopathic effect (Tollefsen et al., 1996a; Subramanian et al., 2006).
From a virotherapy perspective, our aim was to enhance cell lysis, Ad propagation, and antitumor activity of oncolytic Ads. As AAV induced a faster Ad release and increased cytotoxicity, but reduced virus production (burst size), we examined how AAV6 affected ICOVIR15 propagation. With this purpose, propagation-mediated cytotoxicity assays were performed in A549 and NP9 cells using serial virus dilutions (IC₅₀ assays). ICOVIR15 propagated faster in the presence of AAV6 at all doses tested. Earlier progeny release is the most obvious explanation for this rapid propagation, increasing the speed of cell-to-cell spread. Using serial dilutions of infected cells, we also observed a significant IC₅₀ reduction (11-fold) in both cell lines. The use of coinfected cells is of interest as oncolytic viruses have been administered by carrier cells (Willmon et al., 2009). Our results indicate that the lower Ad yields associated to AAV coinfection are compensated by an earlier release of virus to the SN, and the final outcome is a faster Ad propagation and cytotoxicity (lower IC₅₀ values).

To apply our findings to virotherapy, we treated lung and pancreatic adenocarcinomas with a mixture of AAV and oncolytic Ad. It is reported that AAV per se can function as a tumor suppressor (Khleif et al., 1991); thus, an AAV-alone treatment was also included. Coinjection with AAV6 improved the antitumor efficacy of ICOVIR15. The faster Ad release and the lower Ad total production, both induced by AAV coinfection, may pose a challenge when attempting to correlate efficacy with intratumoral virus amount by Ad DNA quantification or capsid staining. In fact, we did not find significant differences between intratumor Ad genomes of ICOVIR15/AAV6 and ICOVIR15 groups (data not shown). The strategy to use AAV to foster Ad oncolysis requires coinfection of tumor cells; to maximize it, we used intratumoral administration of a mixture of viruses. Despite that this administration route is commonly used in virotherapy, ideally, systemic administration would be preferred for metastatic cancer. Systemic administration of both viruses would require an AAV biodistribution study in animals bearing tumors after intravenous administration. However, given the limited tumor-targeting capability of Ad, the probability of coinfection of target cells is likely low. Therefore, novel approximations that allow Ad and AAV codeelivery should be explored. Use of carrier cells would be an option (Coukos et al., 1999; Garcia-Castro et al., 2005; Hamada et al., 2007). Another strategy could use nonviral vectors carrying infectious plasmids encoding both viruses (Kwon et al., 2011). This strategy is feasible as AAV plasmids are infectious when transfected and plasmids with self-excising Ad genomes have been developed (Stanton et al., 2008).

Our results point that contamination of Ad stocks with AAV could result in enhanced oncolysis, contrary to what could be expected considering only the widely accepted lower Ad yields. Besides the implication that intentional mixing of Ad and AAV may have for efficacy, this observation also raises a caution note on oncolytic Ad potency comparisons, particularly by direct intratumoral administration, as it would require prior AAV detection.

Acknowledgments

We thank María José Castro Castro (Biochemistry Service of Bellvitge Hospital) and Adoración Huertas Ruz and Elisabet Cañete Ortiz (Epigenetic and Cancer Biology Program of the Bellvitge Biomedical Research Institute) for facility and technical support; Juan José Rojas (University of Pittsburgh Cancer Institute, Pittsburgh, PA) and Miriam Bazan-Peregrino (Institut d’Investigacions Biomèdiques de Bellvitge, Barcelona, Spain) for extensive revision of this article; and Mercedes Saéz (IASIST, S.A.U., Barcelona, Spain) for statistical assistance.

E.L. was supported by a predoctoral fellowship Formación de Profesorado Universitario (FPU) granted by the Spanish Ministry of Education and Science. This work was supported by a grant from the Spanish Ministry of Education and Science (BIO2011-30299-C02-01) and PLE2009-0115 from Spanish Ministry of Economy and Competitivity, and received partial support from the Generalitat de Catalunya (2009SGR264).

Author Disclosure Statement

No competing interests for any of the authors exist.

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Received for publication August 7, 2013; accepted after revision September 9, 2013.

Published online: September 10, 2013.