Noninvasive Visualization of Adenovirus Replication with a Fluorescent Reporter in the E3 Region

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Abstract

To overcome the inefficacy and undesirable side effects of current cancer treatment strategies, conditionally replicative adenoviruses have been developed to exploit the unique mechanism of oncolysis afforded by tumor-specific viral replication. Despite rapid translation into clinical trials and the established safety of oncolytic adenoviruses, the in vivo function of these agents is not well understood due to lack of a noninvasive detection system for adenovirus replication. To address this issue, we propose the expression of a reporter from the adenovirus E3 region as a means to monitor replication. Adenovirus replication reporter vectors were constructed with the enhanced green fluorescent protein (EGFP) gene placed in the deleted E3 region under the control of the adenoviral major late promoter while retaining expression of the adenovirus death protein to conserve the native oncolytic capability of the virus. Strong EGFP fluorescence was detected from these vectors in a replication-dependent manner, which correlated with viral DNA replication. Fluorescence imaging in vivo confirmed the ability to noninvasively detect fluorescent signal during replication, which generally corresponded with the underlying level of viral DNA replication. EGFP representation of viral replication was further confirmed by Western blot comparison with the viral DNA content in the tumors. Imaging reporter expression controlled by the adenoviral major late promoter provides a viable approach to noninvasively monitor adenovirus replication in preclinical studies and has the potential for human application with clinically relevant imaging reporters. (Cancer Res 2005; 65(22): 10154-8)

Introduction

Conventional cancer treatments, including surgery, radiation therapy, and chemotherapy, have improved little over the years with respect to efficacy and are paradoxically associated with undesirable toxicities. In response to these issues, adenovirus virotherapy has been proposed as a promising alternative to treat tumors in an effective yet safe manner (1) through a lateralizing, oncolytic mechanism that is distinct from all other forms of cancer therapies. Despite the great potential of virotherapy, evaluation in clinical trials has not validated the effectiveness of conditionally replicating adenovirus (CRAd) as a single-agent therapy against cancer (2). In fact, experience from clinical trials has indicated that little is known about the function of oncolytic adenoviruses in vivo. Basic issues with regard to the function of CRAds, including their replication capacity, spreading ability, extent of persistence, and interaction with the host immune system, have yet to be elucidated. Limited understanding of replicative adenovirus operation may in part be attributed to lack of a noninvasive CRAd monitoring system. The ability to assess adenovirus replication in vivo would aid in further development of this intervention for tumor treatment and also provide a means to monitor its application in clinical trials. We hypothesized that adenovirus replication may be monitored via the detection of a fluorescent reporter expressed from the E3 region under the adenoviral major late promoter. Of note, this adenoviral promoter is only active during viral replication (3). In this body of work, we present an adenovirus replication reporter system for the purpose of monitoring CRAds. We show the function of this system in vitro and show that it could be used to detect adenovirus replication in vivo.

Materials and Methods

Vector construction. The E3 reporter vectors (Ad-E3-EGFP F0, F1, and F2) were constructed based on adenovirus serotype 5 (Ad5). To liberate cloning space for the reporter, the various genes in the E3 region were converted to a TTA stop codon by PCR ligation with primer pairs and . The second fragment contained a partial deletion of the E3 gene generated by ligating the two PCR products from primer pairs and . The 12.5K start codon in this fragment was converted to a TTA stop codon by PCR ligation with primer pairs and . The second fragment was deleted of the 6.7K and 19K genes and generated by ligation of PCR products made with primer pairs and , and . The third fragment contained a Sw1 restriction site in place of the deleted RID-6.5K and 14.7K genes and was constructed by PCR ligation of PCR products from primer pairs and , and . The third fragment was constructed by PCR ligation of PCR products from primer pairs and , and . The third fragment contained a Sw1 restriction site in place of the deleted RID-x, RID-β, and 14.7K genes and was constructed by PCR ligation of PCR products made with primer pairs and . The three fragments were cloned into the respective AarII-NdeI region of pMG100 (4), corresponding to Ad5 bp 25,915 to 31,089, to generate the pShuttle-ΔE3-ADP. To facilitate the cloning of reporter genes into the deleted E3 region and subsequent recombination selection, the blunt pShuttle-ΔE3-ADP was cloned into the respective AarII-NdeI region of pMG100 (4), corresponding to Ad5 bp 25,915 to 31,089, to generate the pShuttle-ΔE3-ADP. To facilitate the cloning of reporter genes into the deleted E3 region and subsequent recombination selection, the blunt pShuttle-ΔE3-ADP was cloned into the respective AarII-NdeI region of pMG100 (4)

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resistance gene and a multiple cloning site was cloned into the SwaI site (3' of the ADP gene) of pShuttle-ΔE3-ADP. The final pShuttle-ΔE3-ADP-KanF contained a homologous recombination left arm [Ad5 bp 25,915 (AaII) to 27,981] upstream of the partially deleted 12.5K gene and a right homologous recombination right arm (Ad5 bp 30,884 to the 3' end of the viral genome) downstream of ADP, the multiple cloning site, and kanamycin. The enhanced green fluorescent protein (EGFP) reporter gene from pEGFP-I (Clontech, Palo Alto, CA) was cloned into the Sall site of the shuttle vector in the forward direction to obtain pShuttle-ΔE3-ADP-EGFP-F0. The frame shifted versions (F1 and F2) were constructed from the F0 plasmid by enzymatic cleavage with BamHI and Xbal, respectively (both located in the multiple cloning site upstream of the EGFP gene), blunting, and self-ligation.

The PacI-AaII fragments of each shuttle vector and the SwaI linearized adenovirus backbone (pVK50; ref. 5) were recombined in B5183 using ampicillin and kanamycin double selection to create replicative reporter vectors with intact E1. After confirming correct recombinants, the kanamycin resistance gene was removed by SwaI cleavage and the linearized plasmids were self-ligated. The PacI linearized resultant plasmids were transfected into 111 cells (a kind gift from Dr. Alex J. van der Eb, Leiden University, Leiden, the Netherlands) for initial vector production and then propagated in A549 cells. All viruses were purified by double CaCl2 ultracentrifugation followed by dialysis against PBS with Mg2+, Ca2+, and 10% glycerol. Final aliquots of viruses were analyzed for viral particle titer (absorbance at 260 nm). Ad-E1-CMV-EGFP (6) and Ad5-Δ24/GFP (7) have previously been described. All plasmid constructs and vectors will be provided upon request.

Cell culture. A549 [human lung adenocarcinoma, replication permissive; American Type Culture Collection (ATCC), Manassas, VA] and BNL-1NG-A2 (RALB/c transformed hepatoma, replication nonpermissive; ATCC) cells were maintained according to the protocol of the manufacturer. The cells were incubated at 37°C and 5% CO2 under humidified conditions.

Fluorescence detection in vitro. A549 and BNL-1NG-A2 cells were infected with 10, 1, 0.1, and 0.01 virus particles (vp) per cell of Ad-E3-EGFP F0, F1, and F2, Ad-E1-CMV-EGFP, and Ad-Δ24-EGFP in white opaque 96-well plates (10,000 cells per well, n = 5). The samples were measured daily with a microplate fluorometer (Fluostar Optima, BMG Labotechnologies, Durham, NC).

Viral DNA quantitation. A549 and BNL-1NG-A2 cells were infected with 1 vp/cell of Ad-E3-EGFP F2 in multiple plates corresponding with each day viral DNA was analyzed. Fluorescence of the samples was measured with a microplate fluorometer for the respective day (0, 0.5, 1, 1.5, 2, 4, 6, 8, and 10 days postinfection) and the designated plate was stored at −80°C until analysis. Viral DNA was prepared from the supernatant in the wells (QIAamp DNA Blood Mini kit, Qiagen). Viral DNA copy number was excised and stored at -80°C until analysis. Each frozen tumor was prepared for tumor viral DNA quantitation. The same tissue samples from the above experiment (−300 mg) were further homogenized by using a glass homogenizer with a double volume of radioimmunoprecipitation assay buffer [1% NP40, 0.1% sodium deoxycholate, 0.1% SDS, 10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, and 1 mmol/L EDTA] containing 5 mg/mL aprotinin (1 mmol/L) and phenylmethylsulfonyl fluoride. After 20-minute incubation on ice and pelleting, the supernatant lysates (25 μg) were resolved by SDS-PAGE and then transferred to a polyvinylidine difluoride membrane (Bio-Rad, Hercules, CA). The membrane was probed with a primary anti-EGFP monoclonal mouse antibody (BD Biosciences Clontech, Mountain View, CA) and then a horseradish peroxidase–labeled anti-mouse IgG secondary antibody (Amersham Pharmacia, Piscataway, NJ), followed by chemiluminescence detection (ECL, Amersham Pharmacia). Band densities were quantitated with ImageTool 3.0 (University of Texas Health Science Center, San Antonio, TX). Note that tumor samples 3R and 4L could not be analyzed due to technical difficulty.

Results and Discussion

Adenoviral replication reporter system design. Several key components were considered in our adenoviral replication reporter system. First, EGFP was applied as a reporter because of its potential promoters and therapeutic genes for CRAd design. Second, the EGFP gene was placed in the deleted E3 region because of strong transgene expression from this locale documented in previous studies (10, 11). Of note, expression from the adenovirus E3 region follows a late pattern and depends on viral DNA replication (10). Such expression profile suggests involvement of the major late promoter, which is only active during viral replication and particularly after progression from early to late infection (3), a property that could be exploited to monitor adenovirus replication. Although the E3 region is dispensable during in vitro amplification (12), the E3 ADP has been implicated in effective dispersion of progeny virions late in infection (13), the lack of which has been associated with poor tumor killing (7, 14). As a result, the third element of our adenovirus replication monitoring design involved maintaining ADP expression in the E3 region, which had been deleted of the other nonessential E3 genes (12.5K, 6.7K, gp19K, RID-β and RID-β, and 14.7K). Deletion of E3 liberated ~2.3 kb of cloning capacity to accommodate the imaging reporter as well as potential promoters and therapeutic genes for CRAd design. Finally, the configuration of the EGFP reporter gene in the E3 region was considered, namely its reading frame relative to the upstream and adjacent ADP gene.

Oncolytic capacity of replication reporter adenovirus. We constructed three versions of our ADP-expressing replication reporter adenovirus with the EGFP gene placed in the forward (5' to 3') direction and positioned in three different reading frames (F0, F1, and F2) downstream of the ADP gene. Aside from differences in the reporter configuration, these three vectors were otherwise isogenic and contained the wild-type E1 region. All three viruses showed cytolysis and spreading ability similar to or
Fluorescence monitoring in vitro. The time course expression of EGFP in relation to viral replication was evaluated in vitro. Because of their ability to support efficient human Ad5 replication (7), human lung adenocarcinoma A549 cells were used as an Ad5 replication permissive substrate. On the other hand, chemically transformed mouse hepatoma BNL-1NG-A.2 cells, in which human adenoviruses do not productively replicate (15), were applied as an Ad5 replication nonpermissive substrate. Indeed, all replication-competent reporter viruses showed fluorescence augmentation over time and in a dose-dependent manner during infection of A549 cells (Fig. 1A, top three panels). A nonreplicative control with a CMV-EGFP expression cassette placed in the deleted E1 region yielded no increase in fluorescence and consequently a flat fluorescence curve in A549 cells (Fig. 1A, bottom left). All viruses, regardless of their replication capability in A549 cells, showed the same flat response in BNL-1NG-A.2 cells, supporting the fact that fluorescence amplification requires productive replication. Representative data for Ad-E3-EGFP F2 in BNL-1NG-A.2 cells is shown (Fig. 1A, bottom middle). At higher multiplicities of infection in A549 cells (particularly 10 and 1 vp/cell), the time course fluorescence follows a sigmoidal pattern. This response likely reflects a lag phase wherein a few cells are initially infected, a log phase in which considerable lateralization and widespread infection of neighboring cells occurred, and a plateau phase in which all of the cells were exhausted for replication and consequently killed. Interestingly, the virus designated as F2 showed the strongest EGFP expression relative to F0, F1, and the CMV-EGFP viruses. All of our replication reporter adenoviruses exhibited far superior fluorescence production than the previously reported vector Ad5-Δ24/GFP that contains the GFP reporter under the SV40 promoter in the fully deleted E3 region (Fig. 1A, bottom right; ref. 7).

Correlation of fluorescence detection with viral DNA replication in vitro. To link the function of our reporter system with the underlying level of replication, the extent of viral DNA replication of the F2 virus was compared with vector expression of the fluorescent reporter. Unlike the 2-day delay in fluorescence gain, a sharp increase in viral DNA was observed as early as 24 hours after infection of replication permissive A549 cells. The fact that strong EGFP expression was detected late in infection after the early-intermediate event of viral DNA replication likely reflects the involvement of the adenovirus major late promoter in controlling the E3 reporter expression. Similarly, it has been previously shown that strong reporter expression from the E3 region depends on high viral DNA copy number in the cell (10). In replication-permissive adenoviruses containing the EGFP gene in three different open reading frames, designated as Ad-E3-EGFP F0, F1, and F2. Fluorescence was detected over the course of 10 days. E1-deleted Ad-E1-CMV-EGFP was included as a nonreplicative control. Representative fluorescence data is shown for Ad-E3-EGFP F2 infection of BNL-1NG-A.2 cells (mouse hepatoma cells, replication nonpermissive). Also depicted are the fluorescence results for a previously reported vector Ad5-Δ24/GFP that contains the GFP reporter under the control of an SV40 promoter in the fully deleted E3 region. Note that SDs (bars) are covered by the symbols (n = 5). B, over the course of 10 days, the fluorescence curves (left axis) for A549 (Δ) and BNL-1NG-A.2 (○) cells (n = 4) infected with 1 vp/cell of Ad-E3-EGFP F2 were compared with the underlying level of viral DNA replication (right axis) determined by real-time quantitative PCR using E4 primers [A549 (○) and BNL-1NG-A.2 (○), n = 4].

Figure 1. Detection of E3 EGFP reporter in vitro. A, A549 cells (human lung adenocarcinoma cells, replication permissive) were infected with various amounts [10 (○), 1 (●), 0.1 (△), and 0.01 (□) vp/cell] of the E3 reporter adenoviruses containing the EGFP gene in three different open reading frames, designated as Ad-E3-EGFP F0, F1, and F2. Fluorescence was detected over the course of 10 days. E1-deleted Ad-E1-CMV-EGFP was included as a nonreplicative control. Representative fluorescence data is shown for Ad-E3-EGFP F2 infection of BNL-1NG-A.2 cells (mouse hepatoma cells, replication nonpermissive). Also depicted are the fluorescence results for a previously reported vector Ad5-Δ24/GFP that contains the GFP reporter under the SV40 promoter in the fully deleted E3 region. Note that SDs (bars) are covered by the symbols (n = 5).

Figure 2. Comparison of replication reporter adenovirus fluorescence in vivo with viral DNA replication. A, established A549 (replication permissive, mouse 3) and BNL-1NG-A.2 (replication nonpermissive, mouse 4) tumors on the left and right flanks of athymic nude mice were injected with a single dose of Ad-E3-EGFP F2 (1010 vp in 50 μL) and imaged various days later with a noninvasive fluorescence imaging system. The data are displayed as a pseudocolored fluorescence intensity image overlaid on a brightfield image of the entire mouse body. Below each whole-body picture is an enlarged view of the tumor. The index scale for the fluorescence intensity is shown to the left of the images. The label above each set of images corresponds to the mouse number and the location of the tumor (R, right and L, left). B, after live imaging, the mice were sacrificed and the tumor homogenates were quantitated for adenoviral E4 DNA copy number using real-time quantitative PCR. The total integrated fluorescence intensity over the entire tumor (the product of mean intensity and signal area, RFU × mm2, white columns, left axis) was compared with the quantitative PCR result (black columns, right axis). The labels for each column set correspond to the respective mouse in (A).
A549 cells, the time course augmentation in fluorescence signal corresponded well with viral DNA replication where the log phases of both variables matched during the same time period. Furthermore, the leveling of fluorescence occurred concomitantly with the plateau in viral DNA replication. The results obtained for BNL-1NG-A.2 cells were quite different. Because these mouse cells are nonpermissive for productive human adenovirus replication, the viral DNA replication detected (a little over 1 log increase from the initial amount) was significantly lower relative to that in A549 cells (over 5 log increase), whereas the fluorescence remained unchanged (Fig. 1B). The basal level of viral DNA replication in BNL-1NG-A.2 cells is consistent with previous observations of early replication in mouse cells, including early gene expression and DNA replication but failed transition from early to late infection precluding infectious progeny production (15, 16). Altogether, these in vitro data validate the use of our reporter system for monitoring adenovirus replication.

Comparison of fluorescence detection in vivo. Established A549 and BNL-1NG-A.2 flank tumors in athymic nude mice were administered the F2 vector in a single injection. EGFP expression resulting from viral replication was detected with a noninvasive fluorescence-based optical imaging system. Diverse EGFP expression was noted from tumor to tumor although the F2 virus was administered at the same time and in equal doses (Fig. 2A). After acquiring live images of EGFP intensity and localization, the tumors were excised and homogenized to determine the total amount of adenovirus DNA copy number resulting from replication. The results show that noninvasively detected EGFP expression from the E3 region generally corresponded with the total viral DNA content in the tumor (Fig. 2B) except for the case of tumor 1L. Because tissue depth can greatly affect the detectability of EGFP (17), perhaps leading to this discrepancy, we analyzed the quantity of EGFP in the tumors on a more molecular level by performing a Western blot analysis with the tumor homogenates. The data indicate that the extent of EGFP expression in the tumors indeed correlates very well with the viral DNA copy number (Fig. 3). In contrast to the results obtained for the replication-permissive A549 tumors, no fluorescence signal was detected from the replication-nonpermissive BNL-1NG-A.2 tumors in vivo (Fig. 24),
corresponding with the data obtained from viral DNA quantitation and Western blot analysis (Figs. 2B and 3, respectively). These data, like the results from the in vitro studies, highlight the capacity of our reporter system to represent the underlying level of adenovirus replication in vivo.

Monitoring replication in vivo. We applied our detection system to monitor the time course replication of the F2 virus in vivo in established A549 tumors with live imaging over 19 days. Interestingly, EGFP fluorescence could be detected as early as 2 days postinjection (Fig. 4A). The ability to achieve early detection of adenovirus replication in the clinics would offer the potential to acquire valuable interval end point data with respect to the function of CRAds, a utility not common with conventional vector detection techniques. We were able to follow the progression of the replicating F2 vector, which showed increase in fluorescence in the first week but a gradual decline in signal thereafter, likely representing vector clearance (Fig. 4B). Of note, the area of infected cells did not seem to spread beyond the initial region of positive signal, perhaps explaining the inability of the virus to effectively cause oncolysis of the entire tumor.

We have shown that the EGFP reporter gene placed in the deleted E3 region could be exploited to monitor adenovirus replication. Fluorescence detection correlated with the underlying level of replication both in vitro and in vivo. Our reporter system could be applied to noninvasively monitor the time course progression of adenovirus replication. Although the EGFP reporter was operative in our in vivo experiments, we are cognizant of its major limitation for clinical use, namely poor detectability deep in tissue. Other imaging reporters more suitable for clinical applications should be considered, including thymidine kinase (18), the sodium iodine symporter (19), and the somatostatin type 2 receptor (20). The liberation of 2.3 kb cloning capacity through E3 deletion makes it possible to apply these candidate imaging reporters as well as tissue-specific promoters, fiber modifications, and therapeutic genes for CRAd design. Another important component of our replication reporter system is the fact that ADP expression is maintained to preserve the wild-type oncolytic ability of adenovirus. However, the effect of E3 deletion on the function of CRAds will have to be analyzed in more detail especially with respect to the documented roles of E3 products in immunomodulation. Our reporter system provides a much needed monitoring capability for the development of CRAds as well as for the application of oncolytic adenoviruses in the clinics.

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