Quantifying the Activity of Adenoviral E1A CR2 Deletion Mutants Using Renilla Luciferase Bioluminescence and 3′-Deoxy-3′-[^18F]Fluorothymidine Positron Emission Tomography Imaging

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Abstract

The adenoviral E1A CR2 mutant dl922-947 has potent activity in ovarian cancer. We have used Renilla luciferase bioluminescence imaging to monitor viral E1A expression and replication and [^18F]fluorothymidine positron emission tomography ([^18F]FLT-PET) to quantify the activity of dl922-947 in vivo. We created dlCR2 Ren, with the same E1A CR2 deletion as dl922-947 and the luciferase gene from Renilla reniformis downstream of E1. Light emitted from s.c. and i.p. IGROV1 ovarian carcinoma xenografts was measured following treatment with dlCR2 Ren. Mice bearing s.c. IGROV1 xenografts were injected with 2.96 to 3.7 MBq of [^18F]FLT 48 and 168 hours following i.t. injection of dl922-947 or control virus Ad LM-X. The presence of Renilla luciferase in dlCR2 Ren did not reduce in vitro nor in vivo potency compared with dl922-947. Light emission correlated closely with E1A expression in vitro and peaked 48 hours after dlCR2 Ren injection in both s.c. and i.p. IGROV1 xenografts. It diminished by 168 hours in s.c. tumors but persisted for at least 2 weeks in i.p. models. Normalized tumor [^18F]FLT uptake at 60 minutes (NUV<sub>60</sub>), fractional retention, and area under radioactivity curve all decreased marginally 48 hours after dl922-947 treatment and significantly at 168 hours compared with controls. There was a close linear correlation between NUV<sub>60</sub> and both tumor proliferation (Ki67 labeling index) and thymidine kinase 1 expression. Renilla luciferase bioluminescence and [^18F]FLT-PET imaging are capable of quantifying the activity and effectiveness of E1A CR2–deleted adenoviral mutants in ovarian cancer. (Cancer Res 2006; 66(18): 9178-85)

Introduction

Ovarian cancer prognosis remains poor despite recent advances in chemotherapy (1, 2). Replicating oncolytic viral vectors show promise as novel treatments for cancer. Such viruses infect cancer cells, multiply within them, and cause cell death with release of mature viral particles that infect neighboring cells. However, although some tumor-selective replication has been seen in early-phase clinical trials (3), durable responses to single agent viruses are rare, especially in ovarian cancer (4). dl922-947 is an adenoviral mutant with a 24-bp deletion in the E1A CR2 region, which normally drives infected cells into S phase by disrupting the interaction between host-cell retinoblastoma protein and E2F. Thereafter, viral DNA is replicated in preference to that of the host cell. dl922-947 has been shown to replicate specifically in human ovarian cancer cells but not in ovarian surface epithelial cells with an intact retinoblastoma pathway (5), and to have greater efficacy than either wild-type adenovirus or the E1B-55K deletion mutant dl1520 (5, 6).

Ovarian cancer causes i.p. changes that are difficult to quantify by conventional imaging, such as ascites formation and multiple nodules disseminated on the peritoneal surface. Response assessment in xenograft models by postmortem examination of cohorts of animals is wasteful and generates only static snapshots of data. Using ovarian cancer cells that stably express Firefly luciferase, we have used bioluminescence imaging to monitor tumor growth and response to i.p. dl922-947 (5). However, evaluation of the activity of the virus itself presents significant logistical difficulties. Two recent reports have suggested that fusion of red fluorescent proteins to the adenoviral minor capsid protein IX (7) or insertion of an enhanced GFP gene into the E3 region (8) allows visualization of viral replication in vivo. However, this requires activation of fluorescence with an external light source, which makes imaging i.p. disease difficult. We have now developed an E1A CR2–deleted adenovirus encoding the luciferase gene of the sea pansey Renilla reniformis. This gene uses different substrates to Firefly luciferase and emits light at a different wavelength. Here we show that this virus, dlCR2 Ren, has comparable efficacy to dl922-947 and can be used to quantify viral activity both in vitro and in s.c. and i.p. murine xenograft models.

Another novel technique for evaluating response to treatment is positron emission tomography (PET). The most commonly used clinical PET tracer is [^18F]fluorodeoxyglucose (FDG), which provides information on glucose transporters and hexokinase activity. In advanced ovarian cancer, imaging with FDG-PET is capable of predicting response to neoadjuvant chemotherapy (9). However, FDG is trapped by other tissues, such as macrophages, granulation tissue, and in areas of necrosis, reducing its specificity. Because all anticancer agents ultimately aim to alter tumor cell proliferation, newer PET substrates aim to quantify proliferation rather than glucose metabolism. One such substrate is 3′-deoxy-3′-[^18F]fluorothymidine ([^18F]FLT). Upon entering the cell,[^18F]FLT is trapped following monophosphorylation by cytosolic thymidine kinase 1 (TK1), the activity of which correlates with cellular...
proliferation in cancer (10). Initially, [18F]FLT-PET was used as a

Materials and Methods

Radiochemicals and luciferase substrate. [18F]FLT was produced by

Bioluminescence and FLT-PET for d922-947

Figure 1. dCR2 Ren has similar activity to d922-947 in vitro and in vivo. A, in vitro activity of d922-947 and dCR2 Ren in ovarian carcinoma cell lines. IGROV1 and OVCAR4 cells were infected with d922-947 and dCR2 Ren (MOI 0.001-1.000 pfu/cell). Cell survival was assessed by MTT assay up to 120 hours later. Top, points, mean percentage cell survival compared with mock-transfected cells (n = 3); bars, SD. Bottom, IC50 results. B, in vivo activity of d922-947 and dCR2 Ren in IGROV1 xenograft. Female nude mice were inoculated i.p. with 3 x 106 IGROV1 cells on day 1. On days 4 to 8 inclusive, mice received i.p. injections of Ad LM-X, d922-947, dCR2 Ren (5 x 1010 particles per injection), or an equal volume of vehicle (20% icodextrin); n = 10 per group. Animals were monitored for accumulation of ascites and for general well-being. Kaplan-Meier survival curves are presented. * P = 0.013; ** P = 0.002 (d922-947); P = 0.004 (dCR2 Ren); log-rank test.

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Hammersmith Imanet Limited (Hammersmith Hospital, London, United Kingdom) as previously described (15). All samples had >99% radiochemical purity as determined by radio-high-performance liquid chromatography and the specific radioactivity ranged between 24 and 465 GBq/mmol. The Renilla luciferase substrate was coelenterazine e (Insight Biotechnology, Wembley, United Kingdom), which emits 1.37 times more total light than native coelenterazine, with 7.5 times greater initial intensity in the presence of the Renilla luciferase gene (16).

Cell culture, adenoviral construction, cell viability and viral replication assays, and in vitro luciferase assay. IGROV1 and OVCAR4 were incubated at 37°C with 10% CO2 in air, in DMEM plus 10% heat-inactivated FCS (IGROV1) or in RPMI medium plus 10% FCS (OVCAR4). d922-947 is an Ad5 vector deleted in the region encoding amino acids 122 to 129 of the E1A CR2 domain as well as in E3B (6). dCR2 Ren is also an Ad5 vector deleted in the same amino acids of E1A CR2 and which additionally contains a reverse orientation expression cassette immediately downstream of E1 that encodes the luciferase gene of Renilla reniformis under the control of the herpes simplex virus-1 TK1 promoter. A full description of the construction of dCR2 Ren is found in Supplementary Methods. Schematic representations of dCR2 Ren and d922-947 are shown in Supplementary Fig. S1. The construction of the E1-deleted control vector Ad LM-X has been described elsewhere (17).

For cell viability assays, 2 x 105 cells were infected in triplicate with adenovirus in serum-free medium. After 120 minutes, cells were refed with medium plus 5% FCS. Viability was assessed by trypan blue dye exclusion. To determine cell viability, infected cells were plated at a density of 1 x 104 per well in a 96-well plate and grown to confluence. Cells were then infected with the virus of interest at a multiplicity of infection (MOI) of 10 plaque-forming units (pfu) per cell and harvested up to 72 hours later into 0.5 mL of 0.1 mol/L Tris (pH 8.0). Cells were subjected to three rounds of freeze/thawing (liquid N2/37°C), after which they were centrifuged. The supernatant was titered on J293 cells by serial dilution.

Western blotting. Following adenoviral infection, 5 x 104 cells were scraped into 200 mL of lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.5), 0.05% SDS, 1% Triton X-100] and sonicated on ice. Ten micromolar concentrations of protein were electrophoresed on SDS-polyacrylamide gels and transferred onto a nitrocellulose filter by semidry blotting. Antibody binding was visualized by enhanced chemiluminescence (Amersham Pharmacia, Bucks, United Kingdom).

In vivo analyses, imaging, and histopathology. All experiments were done under suitable UK Home Office personal and project license authority using adult female BALB/c nu/nu mice. In s.c. tumor experiments, 5 x 106 IGROV1 cells were injected. For i.p. experiments, mice were injected with 3 x 106 IGROV1 cells. For overall survival experiment (Fig. 1B), virus was injected daily on days 4 to 8 inclusive [5 x 107 particles in 400 mL of 20% icodextrin (Innovata plc, Nottingham, United Kingdom) per injection]. Mice were assessed daily for weight, general health, and accumulation of ascites.

Bioluminescence imaging. For imaging of s.c. tumors, groups of up to six animals received a single i.t. injection of virus (1 x 1011 particles in 50-µL PBS) or no treatment once tumors reached ~150 mm3. At fixed time points, mice were anesthetized (1.5 mL/kg i.p. of 2:1 ketamine/xylazine) and injected with coelenterazine e (0.5 mg/kg in 100-µL PBS by tail-vein injection). While still under anesthesia, mice were placed in a light-tight chamber on a warmed stage (37°C) and light emission was captured for 3 minutes with a Xenogen IVIS Imaging System 100 system (Xenogen, Alameda, CA). Bioluminescence data from defined regions of interest were analyzed with Living Image software (Xenogen) and are presented as the photon counts per second per square centimeter. Mice were sacrificed and tumors were excised and fixed in 4% formalin. Four-micrometer sections were processed for hematoxylin and eosin staining and stained with a rabbit anti–Ad2 E1A antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

For i.p. experiments, a single dose of virus (1 x 1010 particles in 400 mL of 20% icodextrin) was administered on day 4. Mice were anesthetized (2% halothane by inhalation) and injected i.p. with coelenterazine e (0.3 mg/kg in 200-µL PBS) and imaged on the IVIS machine for 3 minutes.

PET imaging. Once s.c. IGROV1 xenografts reached ~100 mm3, mice were allocated to one of five size-matched groups: imaging with no treatment, imaging 48 or 168 hours after a single i.t. injection of Ad LM-X or d922-947 (1 x 1010 particles in 50-µL PBS). Anesthesia was induced with
Results

dCR2 Ren has comparable activity to d922-947 in vitro and in vivo. We first wished to assess whether the presence of the luciferase gene in dCR2 Ren altered efficacy. The cytotoxicity of dCR2 Ren, as quantified by MTT assay, was similar to that of d922-947 (Fig. 1A); in IGROV1 cells, the IC50 for d922-947 120 hours posttransfection was 0.18 pfu/cell compared with 0.16 pfu/cell for dCR2 Ren, whereas in OVCAR4 cells, these figures were 8.3 and 3.1 pfu/cell, respectively (P > 0.05 (not significant) for both cells). An E1-deleted control virus, Ad LM-X, produced minimal reduction in survival at the same range of MOI (data not shown). In vivo, in the aggressive IGROV1 model of ovarian cancer, median survival for vehicle-treated animals was only 34 days (Fig. 1B), whereas treatment with Ad LM-X prolonged median survival to 44 days (P = 0.013, versus Ad LM-X). Such prolongation has previously been observed (5, 19). Treatment with dCR2 Ren and d922-947 extended survival significantly further; median for dCR2 Ren, 72 days; median for d922-947, 120 days (P = 0.002-0.004, versus Ad LM-X, for both viruses). However, there was no significant difference in survival between animals treated with dCR2 Ren and d922-947.

Luciferase activity following dCR2 Ren infection correlates with E1A expression in vitro. We next investigated how the activity of the luciferase gene in dCR2 Ren correlated with both E1A expression and viral replication. There was a rapid increase in light emission in dCR2 Ren–infected IGROV1 cells 24 hours after infection in vitro, which diminished to baseline over the next 5 days (Fig. 2A). E1A expression closely paralleled this (Fig. 2B), peaking 24 to 40 hours postinfection and diminishing until 72 hours; there were insufficient viable cells to permit protein extraction beyond this time point. d922-947–induced E1A expression had similar kinetics to dCR2 Ren, but there was no change in light emission over 7 days with d922-947. These results indicate that light emission from dCR2 Ren–infected cells is a good surrogate for E1A expression and that the presence of the luciferase gene does not alter E1A expression significantly. Virus released from IGROV1 cells transfected with dCR2 Ren and d922-947 is maximal 48 hours postinfection (Fig. 2C). d922-947 seems to replicate to a greater degree than dCR2 Ren at all time points, although the amount of virus released exceeds the initial input dose of 10 pfu/cell by 48 hours with both viruses.

Bioluminescence imaging. To afford comparisons with subsequent [18F]FLT-PET imaging, we initially studied s.c. tumors. IGROV1 xenografts were injected i.t. with a single dose of dCR2 Ren or Ad LM-X in groups of up to six and imaged 48 and 168 hours later. Tumor volume data (Fig. 3A) indicated a difference in mean relative tumor volumes in dCR2 Ren–treated animals 48 hours postinfection (Fig. 3C). d922-947 is maximal 48 hours postinfection (Fig. 2C). d922-947 seems to replicate to a greater degree than dCR2 Ren at all time points, although the amount of virus released exceeds the initial input dose of 10 pfu/cell by 48 hours with both viruses.

was even more marked at 168 hours (Ad LM-X, 2.02 ± 0.75; d/CR2 Ren, 0.9 ± 0.26; P = 0.0095). There was nonspecific activity in the lungs of mice (Fig. 3B), but light emitted from d/CR2 Ren–injected tumors (5.56 × 10^5 ± 1.489 × 10^5 photons/s/cm^2; mean ± SE; n = 6) was significantly higher than controls at 48 hours (5.858 ± 1.811 × 10^4 photons/s/cm^2; P = 0.028). By 168 hours, this difference was less marked, although still present after i.t. injection of d/CR2 Ren or Ad LM-X (both 1 × 10^10 particles) in groups of five and imaged for 3 minutes following i.p. injection of coelenterazine e. Light emission from control mice injected with d/CR2 Ren and PBS was negligible (<10^4 photons/s/cm^2; data not shown), indicating that d/CR2 Ren induced no spontaneous light emission. As with the s.c. experiments, there was background light emission in control mice following coelenterazine e injection (Fig. 4). Nonetheless, d/CR2 Ren induced a wave of light emission that peaked between 48 and 96 hours after injection and was still significantly higher than Ad LM-X– and d/CR2 Ren–treated controls at 168 hours. There was still evidence of continued light emission at 336 hours (2 weeks). One animal per group was sacrificed at 48 hours, with removal of liver and peritoneum. There were no visible tumor deposits; thus, it was not possible to correlate light emission with E1A expression in tumor cells. However, there was evidence of E1A expression on both peritoneum and liver capsule (Supplementary Fig. S2), suggesting that these vectors infected and replicated in at least some nonmalignant tissues within the peritoneal cavity.

Monitoring d/CR2-947 activity by [18F]FLT-PET imaging. Mice with s.c. IGROV1 xenografts were imaged 48 or 168 hours after i.t. injection of d/CR2-947 or Ad LM-X (both 1 × 10^10 particles) following i.v. injection of 2.96 to 3.7 MBq (80-100 μCi) [18F]FLT. A control group of mice bearing untreated tumors (“No Treatment”) was also imaged. Figure 5A presents the static data for normalized uptake values at 60 minutes (NUV_60), area under radioactivity curve, and fractional retention for the five groups, whereas representative cross-sectional images from mice in each group are presented in Fig. 5B and normalized tumor time-versus-radioactivity curves are given in Fig. 5C. There was no significant difference in NUV_60, area under radioactivity curve, and fractional retention between the No Treatment group and the Ad LM-X–treated mice at either 48 or 168 hours (Fig. 5A). Forty-eight hours after virus injection, there was a small, nonsignificant reduction in NUV_60 for the d/CR2-947 tumors compared with Ad LM-X [Ad LM-X, 1.24 ± 0.13; d/CR2-947, 0.98 ± 0.07 (mean ± SE); n = 4]. However, all variables were significantly lower in the d/CR2-947 tumors by 168 hours compared with Ad LM-X at the same time (Fig. 5A-C).

We then correlated these PET data with physical characteristics of the tumors. Tumors in the Ad LM-X and d/CR2-947 groups were similarly sized at the time of virus injection and were also the same size as the tumors in the Ad LM-X group at the time of sacrifice.
size as the comparator No Treatment tumors [No Treatment, 91.2 ± 30.9 mm³; Ad LM-X, 91.2 ± 14.2 mm³; dl922-947, 96.9 ± 39.6 mm³ (mean ± SD); n = 5]. Forty-eight hours after virus injection, there was no obvious change in tumor volume (Fig. 6A, left), but the dl922-947–treated tumors were significantly smaller by 168 hours [Ad LM-X, 176.4 ± 15.6 mm³; dl922-947, 50.4 ± 7.8 mm³ (mean ± SD); n = 5; P < 0.0001]. dl922-947 induced a significant reduction in cell turnover 48 hours after injection, as determined by LI Ki67, and this was even more marked at 168 hours (Fig. 6A, right). By contrast, Ad LM-X treatment caused no change in LI Ki67 at either time point compared with the No Treatment tumors. The expression of TK1, as determined by Western blot, closely mirrored the PET NUV60 data—there was no significant difference between the No Treatment group and either Ad LM-X group (Fig. 6B). dl922-947 induced a small, nonsignificant reduction in TK1 expression by 48 hours and a much larger, statistically significant reduction by 168 hours [absorbance ratio TK1/actin: Ad LM-X, 1.07 ± 0.44; dl922-947, 0.55 ± 0.25 (mean ± SD); n = 4; P = 0.043]. Association graphs (Fig. 6C) indicated a close linear correlation between NUV60 and both TK1 expression (r² = 0.936) and LI Ki67 (r² = 0.893), but not tumor volume (r² = 0.201; data not plotted). E1A immunohistochemistry shows strong staining 48 hours after dl922-947 injection, again with evidence of expression surrounding areas of central necrosis at 168 hours (Supplementary Fig. S5).

**Discussion**

The ability to monitor the biological activity of novel anticancer agents as well as the changes in tumor behavior they induce is important. We have used two dynamic imaging modalities to monitor E1A CR2–deleted adenoviral vectors in ovarian cancer: bioluminescence as a correlate of viral E1A expression and [18F]FLT-PET as an indicator of cell proliferation. We generated a derivative of dl922-947 encoding Renilla luciferase and first needed to check what effect the transgene had on viral activity. In terms of in vitro cytotoxicity and antitumor activity following i.p. delivery in nude mice, dlCR2 Ren was as effective as dl922-947. Similarly, in the s.c. imaging experiments, both vectors were able to induce a significant reduction in mean tumor volume after direct i.t. injection. Together, these data suggest that the presence of the Renilla luciferase gene has no detrimental effect on overall efficacy of the vector. Replication of dlCR2 Ren in IGROV1 cells was lower than that of dl922-947, the cause of which is uncertain. Although dlCR2 Ren lacks E3 11.6 (ADP), this should not have any effect on intracellular replication, as ADP only affects final cell lysis (20). A reduction in replication has been noted in other vectors with transgenes adjacent to E1 (21) and attempts to augment viral potency by inserting transgenes, such as mda-7, into the E1 and/or E3 regions have met with varying degrees of success (22–24). However, dlCR2 Ren may be able to kill cells at a lower intracellular copy number so that a
reduction in replication is not matched by reduced antitumor activity.

We then determined that dICR2 Ren–induced bioluminescence correlated closely with E1A expression. By placing the Renilla transgene adjacent to E1, we aimed to generate a marker for E1A expression as this gene is the first to be expressed after infection and is an absolute requirement for replication. The kinetics of E1A expression did not differ greatly between the two vectors. One previous report investigated the effect of inserting the Firefly luciferase gene adjacent to the E1A, E2B, and fiber genes. Insertion in the two early gene regions was associated with some reduction in potency, but, for all three sites, luciferase activity correlated in vitro with expression of the relevant viral protein (25). However, this analysis did not extend to in vivo imaging.

We have shown the usefulness of dICR2 Ren in vivo, initially imaging s.c. xenografts to permit comparison with subsequent PET data. Results suggested that E1A expression peaks 48 hours postinjection and decreases by 168 hours. We were able to correlate this with immunohistochemistry, which showed intense E1A staining at 48 hours in a harvested tumor, with reduced expression at 168 hours, at which time two tumors showed a ring of E1A expression surrounding central necrosis, suggesting an expanding wave of virus replication. These E1A kinetics are slower than in vitro, but infection of cells in solid tumors is likely to be uneven compared with tissue culture. Following tail-vein injection of coelenterazine, we observed considerable nonspecific activity in the lungs. Previously, i.v. injection of 0.7 mg/kg native coelenterazine into CD-1 mice produced relatively low background activity (≈3 × 10^7 photons/s/cm^2; ref. 26). Coelenterazine undergoes greater auto-oxidation than the native compound in vitro and following systemic delivery to mice, which leads to higher background light emission, but can still generate ~1 log greater activity in Renilla luciferase–expressing animals than the native compound (27). However, the nonspecific background lung activity seen in our study may limit the use of systemic i.v. coelenterazine administration.

More importantly, for the evaluation of ovarian cancer, we were able to translate this imaging modality to i.p. disease and we believe that this is the first description of direct bioluminescence monitoring of in vivo viral activity. Following i.p. injection of coelenterazine, there was background activity within the peritoneum (≈5 × 10^6 photons/s/cm^2) but light emitted in dICR2 Ren–injected mice was significantly greater for an extended period. Previously, i.p. injection of native coelenterazine has been used to image s.c. xenografts (26), but not i.p. virus or disease. Our data suggest that a single injection of virus causes prolonged E1A expression in nude mice, lasting at least 2 weeks. Because i.p. ovarian carcinoma xenografts do not grow as large masses that can be excised for immunohistochemistry, there are few data on duration of viral protein expression within the peritoneal cavity. We harvested peritoneum and liver from mice 48 hours after virus injection, which indicated that there was definite expression of E1A (and hence replication) within normal, nonmalignant murine ovarian tissue.
tissue. Given the greater tropism of these viruses for human tissue, it is likely that i.p. delivery of these vectors in humans could result in significant off-target infection.

[18F]FLT PET imaging attempts to record changes in cell proliferation. Unlike cytotoxic chemotherapy, dl922-947 initially drives infected cells into S phase (5) before induction of cell death. We had speculated that there might be a transient early increase in [18F]FLT retention following dl922-947 treatment, followed by a reduction as widespread tumor cell death occurred. However, there was a minor reduction in all PET variables at 48 hours, which was much greater at 168 hours. Even with direct i.t. injection of a s.c. xenograft tumor, not all cells will be infected equally, and thus there will not be simultaneous S-phase induction. It remains possible that, in individual cells, [18F]FLT uptake would increase initially, especially as the TK1 promoter contains E2F binding sites (28). However, PET imaging gives only an averaged signal from a whole tumor and here indicated no net increase in S phase at 48 hours. The LIK67 decreased to a greater extent at 48 hours than either TK1 expression or [18F]FLT uptake (13, 14). Here, the good correlation between [18F]FLT uptake and TK1 expression in IGROV1 tumors suggests that the changes previously seen were either specific for RIF-1 tumors or due to complex chemotherapy effects.

In summary, we have successfully used these two novel imaging techniques to monitor both the replication dynamics of an oncolytic adenoviral vector and its effects on proliferation in models of ovarian cancer. These results have immediate clinical resonance as a phase I trial of dl922-947 in women with relapsed ovarian cancer is imminent and we hope that [18F]FLT imaging may be useful in monitoring viral activity in these patients.

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References


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