ORIGINAL ARTICLE

Viral dose, radioiodide uptake, and delayed efflux in adenovirus-mediated NIS radiovirotherapy correlates with treatment efficacy

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We have constructed a prostate tumor-specific conditionally replicating adenovirus (CRAd), named AdSPB_RSV-NIS, which expresses the human sodium iodide symporter (NIS) gene. LNCaP tumors were established in nude mice and infected with this CRAd to study tumor viral spread, NIS expression, and efficacy. Using quantitative PCR, we found a linear correlation between the viral dose and viral genome copy numbers recovered after tumor infection. Confocal microscopy showed a linear correlation between adenovirus density and NIS expression. Radioiodide uptake vs virus dose-response curves revealed that the dose response curve was not linear and displayed a lower threshold of detection at 107 vp (virus particles) and an upper plateau of uptake at 1011 vp. The outcome of radiovirotherapy was highly dependent upon viral dose. At 1010 vp, no significant differences were observed between virotherapy alone or radiovirotherapy. However, when radioiodide therapy was combined with virotherapy at a dose of 1011 vp, significant improvement in survival was observed, indicating a relationship between viral dose-response uptake and the efficacy of radiovirotherapy. The reasons behind the differences in radioiodide therapy efficacy can be ascribed to more efficient viral tumor spread and a decrease in the rate of radioisotope efflux. Our results have important implications regarding the desirable and undesirable characteristics of vectors for clinical translation of virus-mediated NIS transfer therapy.

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INTRODUCTION

Prostate cancer is the second most frequently diagnosed cancer and sixth most frequent cause of cancer death in men worldwide.1 To date, no uniformly curative therapy for metastatic prostate cancer has been developed. In some malignancies, for which existing treatment regimens are not completely effective, suicide gene therapy and virotherapy strategies targeting the tumor-associated genetic alterations represent rational directions for the development of novel therapeutic strategies.2–4

The sodium iodide symporter (NIS) is a transmembrane glycoprotein that mediates uptake of iodide into cells, especially thyroid follicular cells.5,6 The presence of NIS on the basolateral membrane of thyroid cells has been exploited for many years for diagnostic imaging purposes as well as for ablative therapy of benign thyroid disease and differentiated thyroid cancer using radioactive iodide (131I). This non-invasive therapy has proven to be a safe and effective treatment for thyroid cancer, even in advanced, metastatic disease.7,8 In order to extend the use of NIS-mediated radiodiode therapy to other types of cancer, we have successfully transferred and expressed the NIS gene in prostate,9 colon,10 and breast cancer cells,11 both in vivo and in vitro, using adenoviral vectors. Our experience with adenovirus-mediated NIS transfer and radiodiode therapy was confirmed in large animal model and has culminated in the opening of a phase I trial for prostate cancer that is currently accruing patients.12,13

In addition to its therapeutic potential, NIS transfer allows for non-invasive imaging. NIS-mediated imaging has been used to: confirm viral infection and NIS gene expression and function,14 quantitate intratumoral (IT) radioisotope uptake in vivo,15 monitor ectopic expression of the NIS gene in vivo in biodistribution studies,16 and to determine optimal therapeutic delivery timing.5 Groot-Wassink et al.17 correlated NIS-mediated radioiodide uptake to viral-injected doses and NIS mRNA expression by imaging. Although a linear correlation between positron emission tomography scanning and postmortem γ counts in healthy liver was found, the correlation between viral dose (and NIS mRNA) to radioiodide uptake was non-linear. Thus, the relationship between NIS-mediated radioiodide uptake and the viral dose injected in tumors is unclear. In addition, a clear relationship between radioiodide uptake and efficacy has not yet been established.

We have developed a prostate-specific, conditionally replicating adenoviral vector that also harbors the NIS gene.9 In this conditionally replicating adenovirus (CRAd) AdSPB_RSV-NIS, the transcriptional control of the E1A gene is governed by a composite probasin promoter targeting prostate cells in order to reduce extratumoral toxicity by induction of tumor selective replication and cell lysis. NIS expression allows for non-invasive imaging and reporting as well as the potential for radiodiode-mediated therapy. This combination of virus-mediated oncolysis and NIS-mediated radioiodide therapy has been termed by Dingli et al.18 as ‘Radiovirotherapy’.

In this report, we have used a xenograft model using LNCaP cells in nude mice which are transduced in vivo with the
Ad5PB_RSV-NIS CRAd. Confocal microscopy and SPECT/CT (single-photon emission computed tomography/computed tomography) NIS imaging were used to analyze tumor viral spread. We have also established radioiodide uptake vs viral dose-response curves and have correlated our imaging studies to radiovirotherapy treatment efficacy. The results show that the outcome of radiovirotherapy is greatly dependent upon viral spread within the tumor. Moreover, we show that the radioiodide uptake vs viral dose-response curve is complex, steep, and limited by a lower and an upper plateau of uptake. We also present evidence indicating that the upper imaging threshold further defines a minimal viral dose for effective radiovirotherapy. We have found that radioiodide efflux and viral spread are the major components in the treatment outcome. We discuss how our results impact upon the clinical translation of virus-mediated NIS transfer therapy.

RESULTS

Correlation between injected and viral dose delivered in IT injection

In order to measure the number of viral genome copies that are actually present within the tumor after IT injection, we performed quantitative PCR assays of tumor samples after virus injection. LNCaP xenograft tumors established in nude mice were infected with a single dose of Ad5PB_RSV-NIS at $10^7$, $10^9$, or $10^{10}$ vp (virus particles). Three days post-injection, animals were killed and the tumors were tested for viral genome copy contents. Each tumor was assayed in triplicate. Figure 1 shows a linear correlation between the viral dose injected and the number of viral genome copies recovered. Thus, at a dose of $10^{11}$ vp, $1.88 \times 10^7$ genome copies per mg of tumor were recovered. Because the ratio of vp to pfu (plaque-forming units) in our preparation is 100:1, this result indicated that approximately 1 in 50 pfu of injected virus was detectable in the tumor at day 3.

Correlation between viral presence and NIS expression

We delivered the Ad5PB_RSV-NIS CRAd IT to four different mice at a dose of $10^{11}$ vp. On days 3 and 10, two mice were killed and their tumors removed and frozen after immersion in OCT (optimal cutting temperature). Four μm-thick slide sections obtained from the tumors were stained for Ad5 hexon (fluorescein isothiocyanate (FITC)) and NIS (Texas Red). Uninfected tumors were also processed similarly and used as controls. At least 25 photographs spanning the whole slides were taken from each tumor. No signal was detected in the control tumors (Figure 2a). Clear Ad5 foci can be seen on infected tumors both at days 3 (Figure 2b) and 10 (not shown), indicating persistence of virus infection. The virus was spread throughout the tumor as it was found in 80 out of 82 (98%) of the images analyzed. NIS expression was restricted to areas of virus infection (Figure 2b). Both hexon and NIS signal were quantitated by counting the intensity of green and red pixels in each slide. The values obtained for days 3 and 10 were plotted against each other (Figures 2c and d). A linear correlation between green pixels (hexon) and red pixels (NIS) was found that was virtually identical between the two time points ($r^2 = 0.73$, slope = 1.23). These data indicate that NIS expression was proportional to the virus load within the tumor and this correlation was independent of time.

Viral dose response measured by in vivo NIS imaging

Having determined that the correlations between viral dose injected and tumor virus load and between tumor virus load and NIS protein expression were both linear, we next studied how the level of NIS-mediated radioisotope uptake varied as a function of viral dose. When LNCaP xenografted tumors reached approximately 200 mm³, a single dose of Ad5PB_RSV-NIS at $10^7$, $10^9$, $10^{10}$ or $10^{11}$ vp was administered IT. NIS-mediated $^{99}$Tc uptake was visualized and quantitated using micro-SPECT-CT imaging. Figure 3a shows outline of the radiotracer in the stomach and the thyroid due to native NIS expression as well as in the bladder as a result of tracer clearance in the urine.19 Figure 3a also shows that virus-mediated NIS expression resulted in tumor uptake of $^{99}$Tc and that the strength of the signal increased with viral dose. To further ascertain the nature of NIS expression as a function of the viral dose, we quantified the levels of $^{99}$Tc uptake as a function of the concentration of virus delivered into the tumor. At days 1, 2, and 3 post-injection, three mice per dose were analyzed. At each time point, 0.5 mCi $^{99}$Tc was administered intraperitoneally (i.p.), and the uptake was visualized and quantified by micro-SPECT-CT imaging. The results are shown in Figure 3b. For each dose, uptake remained fairly constant throughout the duration of the study except for the $10^{10}$ vp dose in which a small linear increase was detected. Although at all doses tested we observed that NIS expression mediated $^{99}$Tc uptake above that seen in the uninfected control, surprisingly the level of uptake varied only very slightly between the doses of $10^7$ and $10^{10}$ vp, then increased threefold at $10^{11}$ vp. The day-3 dose response curve was plotted and demonstrated a steep increase in radiotracer uptake between the doses of $10^6$ and $10^{11}$ vp (Figure 4a).

Because we showed in Figure 1a linear relationship between dose injected and the number of viral genome copies, we plotted the dose response curve as a function of genome copies per mg of tumor. This curve was plotted and fitted for viral doses between $10^7$ and $10^{11}$ vp. For the dose interval analyzed, the dose response curve could only be fitted by a very steep logistic function as shown in Figure 4b. This result indicated that measurable NIS-mediated uptake has a lower threshold of detection at approximately $10^7$ vp and an upper plateau of uptake at $10^{11}$ vp.

Correlation between viral dose response and efficacy

We have shown previously with this vector that the combination of radiotherapy and cytolytic virotherapy was superior to virotherapy alone.1 In light of the finding of a very steep virus dose response with a lower threshold and an upper plateau, we wished to determine the effect of this phenomenon on the efficacy of radiovirotherapy. To address this question, LNCaP xenografts were established in seven groups of mice (average group size $n = 10 \pm 3$). One group of mice was used as control (C),
four groups received a single IT dose of AdSPB_RSV-NIS at $10^9$, $10^{10}$, or $10^{11}$ vp each (virotherapy V), and the following four groups received each a single IT injection of AdSPB_RSV-NIS at the aforementioned vp doses, and 4 days later, a single ip. dose of 3 mCi $^{131}$I. The survival of each group was determined using Kaplan–Meier curves (Figure 5). The survival times were subjected to a Cox proportional hazards survival regression model (Figure 5).

Treatment of tumors with AdSPB_RSV-NIS at a dose of $10^9$ vp resulted in no significant difference compared with the untreated control cohort. A modest improvement in the outcome was observed when radioiodide was also administered, but this difference was not statistically significant when compared with the control group. A therapeutic effect was observed at a viral dose of $10^{10}$ vp. However, at this viral dose, radioiodide treatment did not improve efficacy of the treatment. Compared with $10^{10}$ vp plus or minus radioiodide, no significant differences were observed at a dose of $10^{11}$ vp. However, when radioiodide therapy was combined with virotherapy at a dose of $10^{11}$ vp, a major improvement on the outcome of the treatment was observed (Figure 5). Thus, the observed radioiodide viral dose-response uptake (Figure 4) correlated with the efficacy of radiovirotherapy.

**DISCUSSION**

We have reported the use of prostate-targeted CRAds expressing the NIS protein in in vivo mouse models of prostate cancer.°
We have shown in this report that measurable NIS-mediated radioisotope uptake has a lower threshold of detection at $10^7$ vp and an upper plateau of uptake at $10^{11}$ vp that correspond to $1.88 \times 10^7$ genome copies per mg of tumor, with a very steep viral dose-response curve. The observed radioiodide viral dose-response uptake correlates well with radiovirotherapy efficacy. Thus, we showed that radioiodide therapy is only effective when approximately $10^7$ genome copies per mg of tumor were present within the tumor. At these higher doses, the virus was also dispersed more homogeneously within the tumor. NIS expression was linearly correlated to hexon expression with a Hexon:NIS ratio of ~1.2, indicating that almost all infected tumor cells expressed NIS.

As shown by the pinhole imaging studies, at an injected dose of $10^{11}$ vp, robust NIS expression covering the entire tumor was observed. By contrast, at lower viral doses effective NIS expression was limited to discrete foci within the tumors. It is likely that, at these low viral doses, tumor cell killing would be restricted to isolated regions within the tumor, whereas at higher doses, the killing would be extended to larger areas of the tumor. NIS can mediate robust bystander effects through $^{131}$I decay $\beta$ particle emission that can kill cells within a 2-mm radius. By homogeneously spreading the virus throughout the tumor, a more effective bystander effect is likely. Our confocal microscopy results showed that although the virus was distributed over the entire tumor, not all cells were infected with Ad5PB_RSV-NIS. However, homogeneous viral distribution will result in overlapping ‘killing zones’, resulting in larger areas of destruction of the tumor mass.

A second consequence of a better viral dispersion and NIS expression is an increase in the $t_{1/2}$ of radionuclide efflux. The $t_{1/2}$ parameters are dependent on the values of clearance and volume of distribution and increased $t_{1/2}$ translates into a better...
A major limitation to viral spread is the high interstitial fluid pressure in solid tumors. This limitation may be overcome by improving either the method of viral infusion or by improving the replicating capabilities of the virus or both. Until better methods of virus delivery are developed, virus replicating capabilities and/or virus targeting will be the limiting factor(s) to viral spread. Thus, a rational approach should be undertaken in the design of viral vectors to improve these factors. In addition, for viruses destined to treat metastatic disease, modifications that alter liver tropism must also be an important consideration. For example, adenoviruses that contain modifications of important genes involved in replication, such as Ad-ONXY-015, should be avoided in favor of transcriptional-dependent regulation because modifications of replication controlling genes can result in significant virus attenuation that seem unlikely to meet clinical needs. In addition, viral genes, such as the E3 adenoviral death protein gene, that have a role in adenoviral dispersal may be retained where possible to enhance viral spread. Placing the NIS gene under the control of a potent promoter, as in our current construct, also results in stronger NIS expression. Finally, research on novel adenovirus serotypes and/or shielded viral capsid is also important as is that of serotypes that either de-targets the virus from liver sequestration and/or have low seroprevalence, thus reducing the effects of pre-existing immunity.

In conclusion, we present data that corroborate the efficacy of radiovirotherapy of non-thyroid malignancies. Our results, however, point out to the need for well-designed vectors capable of efficiently spreading within the tumor and resulting in robust NIS expression.

**MATERIALS AND METHODS**

**CRAd construction**

The structure AdSPB_RSV-NIS CRAd is as described elsewhere.

**Cell culture**

The androgen-dependent (LNCaP), prostate cancer cell line was cultured as described.

**Animal experiments**

Experimental protocols were approved by the Mayo Foundation Institutional Animal Care and Use Committee. All animals were purchased from Harlan Laboratories (Indianapolis, IN, USA) and maintained in the Mayo Foundation animal barrier facilities.

**Subcutaneous tumor model**

Xenografts derived from the LNCaP cell line were established into the right flanks of 4–6-week-old athymic nude Foxn1nu mice (Harlan Laboratories) by subcutaneous injection of 4 × 10⁶ cells resuspended in 0.125 μl media and 0.125 μl of BD Matrigel basement membrane matrix (BD Biosciences, Bedford, MA, USA). Mice were maintained on a low iodine diet and T4 supplementation (5 mg l⁻¹) in their drinking water throughout the duration of the experimentation to maximize radiisotope uptake in the tumor and reduce uptake by the thyroid. The mice were examined daily for tumor development.

**Viral injection**

The virus was diluted in saline to the appropriate vp concentration. The volume of injection was 100 μl. Aliquots of the viral solution were deposited in three tumor regions. No pressure was applied for the injection other than normal thumb pressure.

**Titration of IT virus by quantitative PCR**

LNCaP xenografts were established as described above. When tumors reached approximately 200 mm³, they were injected with AdSPB_RSV-NIS
at 10^7, 10^9, or 10^11 vp. Three days post-infection, mice were killed and the tumors harvested. Three tumor samples weighing 30 mg each were taken from each tumor in each injection group, and DNA was extracted using the DNEasy tissue kit (Qiagen, Valencia, CA, USA) with overnight lysis of tumor tissue in ATL buffer/Proteinase K. Each sample was assayed for Ad5PB_RSV-NIS genome copies using the Applied Biosystems TaqMan Universal PCR System according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA, USA). Briefly, reactions contained 300 nM of a forward primer GACGGCTACGACTGAATG from nt27837 to nt27854 in the Ad5 genome, 300 nM of a reverse primer complementing RSV sequences 118 bp upstream of the RSV promoter, CCGCTTTTCGCCTAAACACAC, and 250 nM of a TaqMan probe binding to the forward strand of the junction between Ad5 E3 sequences and the RSV sequences, 6-FAM – ATATCTGGCCCGTACATCGCAGAT – Iowa Black FQ, (Integrated DNA Technologies, Coralville, IA, USA). In all, 5 µl of template DNA was amplified in 25 µl reactions using ABI Prism 7900HT Real Time PCR System (Applied Biosystems). Genome copies per milligram of tissue were quantitated using a standard curve generated by amplification of known quantities of viral genomes.

Confocal microscopy
A total of four mice were subcutaneously engrafted with LNCaP as described previously. Mice received a single IT dose of Ad5PB_RSV-NIS at 10^11 vp. At days 3 and 10, two mice were killed and the tumors removed. Control mice were injected with saline. All tumors were immersed in OCT compound, frozen, and sectioned to 4 µm. Mounted sections were selected from random locations spanning the whole tumor. Immunofluorescence staining was performed as previously described with some modifications.39 Briefly, sections were fixed in 3% formalin and permeabilized with a 0.5% Saponin solution (phosphate-buffered saline, 50 mg/ml normal mouse serum, and 0.5% Saponin) for 10 min on ice. Before human NIS antibody (mouse IgG 1:2000 dilution) treatment, slides were fixed and then blocked with 10% mouse serum. After washing with phosphate-buffered saline, slides were re-blocked with 10% donkey serum and NIS expression was revealed with a Texas red-conjugated donkey Anti-mouse IgG (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Sections were washed five times and blocked with 5% normal goat serum. Adenovirus hexon protein was stained with a goat polyclonal Adenovirus hexon protein conjugated with FITC (1:100; Abcam Inc., Cambridge, MA, USA). Sections were washed with phosphate-buffered saline four times and 100 µl of VECTASHIELD HardSet Mounting Medium with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA, USA) was added as a counterstain and antifade agent. Texas red/FITC-stained cells were analyzed on an LSM 510 confocal laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY, USA). Texas red/FITC-stained cells were analyzed on an LSM 510 confocal laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY, USA). Texas red was excited with a 543 nm helium/neon laser. Emission for Texas red was selected with a 660–615 nm band pass filter. FITC was excited at 488 nm using an argon laser. Emission for FITC was selected with a 505–550 nm band pass filter. DAPI was excited at 364 nm using an argon laser. Emission for DAPI was captured through a 385–470 nm band pass filter. Images were collected at 512 × 512 pixels resolution using a × 40 C-achromat water immersion lens (1.2 n.a.) (Carl Zeiss, Inc.). Four stained sections from each of the time points listed above were analyzed by acquiring at least 25 images in each of the tumor slices. Adobe Photoshop CSS digital imaging software (Adobe Systems Incorporated, San Jose, CA, USA) was used to quantify the density of red pixels (human NIS) and green pixels (adenovirus hexon protein) in each individual image as described.40

Figure 6. Pinhole imaging and radioisotope efflux. LNCaP xenografted tumors were infected with Ad5PB_RSV-NIS at 10^10 or 10^11 vp. Three days post-infection, an injection of 0.5 mCi 99Tc was given IP to all mice. Pinhole images were captured using a non-invasive micro-SPECT-CT imaging system 1 h after radioisotope administration. Images were quantified using the PMOD Biomedical Image Quantification and Kinetic Modeling Software (PMOD Technologies). (a) Tumors injected with 10^10 vp. (b) Tumors injected with 10^11 vp. (c) Radioisotope efflux. Each point was done in triplicate.
Non-invasive imaging of xenografted tumors: parallel hole and pinhole
When engrafted LNCaP tumor size reached 200 mm³, mice were randomly selected for each of the four viral dosing groups (n = 3) and given one IT injection of AdSPB-RSV-NIS CRAD at 10⁷, 10⁸, 10⁹, or 10¹¹vp, respectively. Two mice were injected with saline as a control group. In vivo micro-SPECT-CT imaging was performed 1 h after i.p. injection of 0.5 mCi of ¹³¹I. Animals were anesthetized using a Summit Medical Anesthesia Machine (Summit Medical Equipment Company, Bend, OR, USA). Oxygen (2 LPM) was used throughout induction and exam. Induction was performed with 4% isoflurane using an induction chamber. Mice were kept anesthetized throughout the full measurement by 2% isoflurane using a nose cone. Images were acquired using a Gamma Medica XSPET system (Gamma Medica, Inc., Northridge, CA, USA). The SPECT scan was performed with a low-energy, high-resolution parallel-hole collimator and a single bore, low-energy, 1 mm aperture pinhole collimator. For the parallel-hole scans, a field of view of 12.5 cm, with a reported resolution of 1–2 mm was used. Sixty four projections, 10 sec/projection, were acquired with a total acquisition time of 13:46 min. For pinhole imaging, the radius of rotation was 5 cm giving a 6.84-cm field of view. Sixty four projections were acquired at 80 kVp and 0.28 mA with a slice thickness of 50 µm and a reported resolution of 43 µm. Images were analyzed using the PMOD Biomedical Image Quantification and Kinetic Modeling Software (PMOD Technologies, Zurich, Switzerland). The level of ¹³¹I uptake by the tumor was expressed as tumor activity in µCi/tumor volume (cc).

Efficacy studies
Mice engrafted with LNCaP as above were divided in groups randomly (average group size n = 10 ± 3). The average tumor size at time 0 was 125 ± 30 mm³. One group of mice was used as control (C), four groups of mice received a single IT dose of AdSPB-RSV-NIS at 10⁷, 10⁸, 10⁹, or 10¹¹vp (virotherapy V), and four groups received a single IT injection of AdSPB-RSV-NIS at the indicated vp and 4 days later a single i.p. dose of 3 mCi of ¹³¹I. Tumor volume was measured twice weekly and mice were killed as they met euthanization criteria established by Mayo Foundation Institutional Animal Care and Use Committee.

Radioisotope efflux studies
LNCaP xenograft bearing mice were divided into two groups randomly (average group size n = 3). One group of mice received a single IT dose of AdSPB-RSV-NIS at 10¹² vp; the second group received a dose of 10¹¹ vp. Three days post injection, a single i.p. dose of 0.5 mCi of ⁹⁹Tc was administered. At time intervals, mice were imaged and the images quantified as described above.

Statistics
All curve fittings were performed with SigmaPlot 10.0 (SigmaPlot Software, San Jose, CA, USA). Survival was determined using Kaplan–Meier curves. The survival times were subjected to a Cox proportional hazards survival regression model.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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