Targeted Radionuclide Therapy Using a Wnt-Targeted Replicating Adenovirus Encoding the Na/I Symporter

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Abstract

Purpose: The Na/I symporter (hNIS) promotes concentration of iodine in cells. In cancer gene therapy, this transgene has potential as a reporter gene for molecular imaging of viral biodistribution and as a therapeutic protein promoting 131I-mediated radiotherapy. Here, we combined the imaging and therapeutic potential of hNIS in an oncolytic adenoviruses targeting colorectal cancer cells.

Experimental Design: We generated an adenovirus (AdIP2) encoding hNIS and capable of selective replication in colorectal carcinoma cells. The selectivity of this virus was verified in vitro and in vivo. Its spread in tumors was monitored in vivo using single-photon emission computed tomography/CT imaging upon 99mTcO4− injection and confirmed by immunohistochemistry. Metabolic radiotherapy was done through injection of therapeutic doses of 131I.

Results: We showed in vitro and in vivo the selectivity of AdIP2 and that hNIS expression is restricted to the target cells. Imaging and immunohistochemical data showed that viral spread is limited and that the point of maximal hNIS expression is reached 48 hours after a single intratumoral injection. Administration of a single therapeutic dose of 131I at this time point led to a dramatic reduction in tumor size not observed in hNIS-negative viruses.

Conclusions: This report showed for the first time that the combination of the imaging and therapeutic potentials of hNIS can be applied to oncolytic adenoviruses in experimental models of cancer. (Clin Cancer Res 2009;15(21):6595–601)
Translational Relevance

This study investigated the imaging and therapeutic potential of the Na/I symporter in the context of an oncolytic virus targeting colorectal carcinoma cells. The results showed that single-photon emission computed tomography/CT imaging can be used to visualize viral propagation in tumors. This technology provides unique information on the peak of Na/I symporter expression in the tumor and can be exploited to determine the optimal timing for 131I-mediated radiotherapy. Therefore, this study validates the concept of targeted radionuclide therapy in the context of an oncolytic adenovirus.

of differentiated thyroid carcinomas with 131I (13). Iodide uptake can also be achieved by gene transfer of hNIS, and this expression can be imaged noninvasively in preclinical models (12) and in humans (14). Different modalities have been reported to image hNIS-expressing tissues. They include positron emission tomography, scintigraphic imaging, and single-photon emission computed tomography (SPECT). The imaging of hNIS-expressing tissues is particularly versatile because hNIS can promote cellular uptake of different radioisotopes: 123I- (SPECT), 124I- (positron emission tomography), 99mTcO4- (SPECT), and 111In- (scintigraphic imaging refs. 5, 12, 15, 16). In addition to its imaging potential, NIS has a well-established therapeutic potential (13). In this field, the use of 131I is a well-established approach that has been extensively applied to nonthyroid tumors through hNIS gene transfer in preclinical models (15, 17–21).

The high prevalence of mutations activating the Wnt signaling pathway in colorectal cancer (22) makes it an attractive target for therapeutic interventions, and a series of adenoviruses capable of selective replication in cells with a constitutive activation of this pathway has been described (4, 8, 23, 24). In the present study, we have generated an adenovirus selectively replicating in cells with a constitutive activation of the Wnt-signaling pathway and encoding hNIS, as well as all the relevant controls. Using these reagents, we have examined whether the imaging and therapeutic potential of hNIS could be exploited in the context of Wnt-selective adenoviruses.

Materials and Methods

Generation and production of a Wnt-selective adenovirus encoding NIS. The plasmid pKpH1 encodes the genome of a Wnt-specific adenovirus in which TCF-4 binding sites are present in E1A, E1B, and E4 of the viral genome (8). Insertion of the NIS coding sequence (27) in the gp19k of this adenovirus was done as previously described (5), using homologous recombination in yeast (25). Virus were produced and titrated as previously described (26).


AdP1: Wild-type adenovirus type 5 with hNIS cDNA inserted in the gp19k region of the genome. AdP2: Adenovirus selectively replicating in cells in which the Wnt signaling pathway is constitutively activated and encoding NIS. AdP3: Wild-type adenovirus type 5 in which the gp19k gene has been deleted (control of AdP1). AdP4: Adenovirus selectively replicating in cells in which the Wnt signaling pathway is constitutively activated and in which the gp19k gene has been deleted (control of AdP2).

Cell lines. The cell lines used in this study, their culture, and their infection were previously described (28, 29). The status of activation of the Wnt-signaling pathway in the cell lines used was previously defined (30).

Biochemical assays. Cell survival assays and assessment of in vitro viral replication and E1A expression were done as previously described (5, 6, 28). H&E staining and hNIS immunohistochemistry were done as previously described (27). Iodine uptake and Western blots were done as previously described (28, 31).

Animals. All experiments were conducted with appropriate ethical approval and accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (House of Commons 1990). Six- to eight-week-old BALB/c nu/nu mice were obtained from Harlan (UK). HCT116 cells were grown as xenografts by injection of 2 x 106 cells s.c. in the right flanks of BALB/c nu/nu mice.

Time-course of iodide retention in the tumor. The biodistribution of iodide was measured as previously described (31).

NanoSPECT/CT imaging. Animals were anesthetized and then given an i.v. injection of 18.5 MBq of 99mTcO4-. Mice were then placed onto a sealed prewarmed (37°C) mouse bed of the SPECT/CT scanner (Bioscan, Inc.) connected to gas anesthetic. The scans started exactly 10 min after 99mTcO4- injection. Mice were imaged as follows. A tophogram of the entire mouse bed was acquired to determine the limits of the SPECT/CT scan of the mouse to be taken. The CT scan was then acquired first, followed by the SPECT acquisition using the limits set by the tophogram. The time of acquisition of the CT scan depended on the size of the scan (as determined by the topographical limits of the scan), the time of the SPECT acquisition depended on the total activity of the radiotracer injected, and the size of the scan (as determined by the topographical limits). In any case, the total scanning time (SPECT and CT) never exceeded 30 min. SPECT acquisition collected 100,000 counts per projection, using a 16 projection and four pinhole system. SPECT and CT scan data were reconstructed using the MEDIso software package (Medical Imaging Systems). SPECT and CT images were coregistered and fused using PMOD software (PMOD Technologies). Quantification of radioisotope accumulation was carried out using In VivoScope software (Medical Imaging Systems) by drawing voxel-guided specific volumes of interest using the fused SPECT/CT images. To account for variations in the activity injected, quantification of radioisotope accumulation in the tumor was expressed as the total amount of radioactivity in the tumor (megabecquerel) divided by the radioactivity measured in the stomach (megabecquerel). In the kinetic study presented in Fig. 4, this process (anesthesia, 99mTcO4- injection, data acquisition, quantitation, and analysis) was repeated every day.

131I-mediated radiotherapy. Six- to eight-week-old BALB/c nu/nu mice bearing HCT116 cells tumors (on average 1 cm2 in size; no statistically different between the different groups using a two-tailed ANOVA test) were injected with 103 plaque-forming units (pfu) of AdP2. Forty-eight hours later, a single dose of 131I (1.5 mCi) was administered i.p. Mice were fed i-thyroxine (5 mg/ml) in their drinking water 1 wk before radioactive iodide administration to suppress thyroid iodine uptake. The larger (L) and smaller (S) size of the tumor were measured at different days, and the tumor size is expressed in square centimeters (L x S).

Statistical analysis. Statistical analysis was done using Prism (GraphPad software). Dual comparisons were made using the Student’s t test, and comparison between multiple conditions was analyzed using ANOVA.

Results

Cytotoxicity in vitro: selectivity of AdP2. To evaluate the impact of the modification of the E3 region of the adenoviral
genome (deletion or insertion of hNIS cDNA), the cytotoxic effects of AdWt, AdIP1, and AdIP2 were examined in target (Wnt activated) and nontarget (Wnt nonactivated) cells. Supplementary Fig. S1 shows that AdIP2 is two orders of magnitude less potent than AdWt in nontarget HeLa cells and unable to induce cytotoxicity in SKBR3 and MCF-7 cells. By contrast, AdWt and AdIP2 were equipotent in most target cells, and AdIP2 was even more efficient than the wild-type virus in SW480 cells ($P < 0.05$; Supplementary Fig. S2). These data suggest that the AdIP2-cytotoxicity is selective to cells with a constitutively activated Wnt signaling pathway.

**Deletion and/or insertion of NIS in the of gp19k locus: impact on the cytotoxicity in vitro.** In all cell lines tested, AdIP1 was consistently the least potent virus (Supplementary Fig. S3), suggesting that insertion of hNIS in the adenoviral genome is detrimental to the cytotoxic activity in vitro. This observation was confirmed in the Wnt-selective viruses, in which AdIP4 was found consistently more potent than AdIP2 in target cells (SW480, SW620, and HCT116 for the three cell lines; $P < 0.05$; Supplementary Fig. S4). The deletion of gp19k did not significantly affect the cytotoxic potential of the virus, except in HeLa and SKBR3 cells, in which this deletion seems to be clearly detrimental in two cell lines (AdWt versus AdIP1 on Supplementary Fig. S3). Overall, these data show a clear detrimental effect of insertion of hNIS in the E3 locus.

**Expression of hNIS in vitro.** HeLa or SW620 cells were infected with Ad-hNIS, AdIP1, AdIP2, and AdIP4. Immunocytochemistry 48 hours after infection shows that hNIS is expressed in Ad-hNIS-, AdIP1-, and AdIP2-infected SW620 target cells, but this expression is absent in AdIP2-infected HeLa cells. As expected, infection of either cell type with the hNIS-negative AdIP4 resulted in a lack of expression of hNIS (Fig. 1A). Functional studies done on SW480 cells infected with Ad-hNIS, AdIP1, and AdIP2 showed that the iodine uptake is dependent on the viral dose and is sensitive to the hNIS inhibitor perchlorate (Fig. 1B). These data show that hNIS expression from AdIP2 is functional, and its expression is restricted to target cells.

**Replication in vitro.** SW480 cells were infected with AdWt and AdIP2 at low multiplicity of infection, and total DNA was extracted. Data from E1A-specific quantitative PCR shows that the viral DNA content of the cells increases with time, suggesting replication (Fig. 2A). To show that replication leads to production of infective particles, HCT116 target

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**Fig. 1.** Na/I symporter expression upon infection. HeLa, SW480 or SW620 cells were infected at different MOIs with the different viruses. Twenty-four (A) or forty-eight (B) hours later, cells were subjected to immunocytochemistry to detect hNIS expression (A) or to a functional assay to measure iodine uptake (B). P stands for the NIS inhibitor sodium perchlorate (100 μM). The data presented are representative of two ($×$300) (A) and are means ±/− SEM of triplicates and are representative of three independent experiments (B).
cells were infected with AdWt, AdIP1, AdIP2, and AdIP4, and the viral content of the cellular homogenates was titrated. Figure 2B shows that infection with all four viruses is capable of producing viral progeny in vitro, with AdWt and AdIP4 infection leading to substantially more virus production than AdIP1 and AdIP2 (P < 0.01). These data suggest that, as for the cytotoxicity in vitro (Supplementary Figs. S1-S4), the presence of hNIS in the viral genome decreases the potential of viral production.

**Selectivity and toxicity in vivo.** AdIP1 and AdIP2 (10^9 pfu) were injected i.v. into BALB/c nude mice. Livers were collected 48 or 120 hours later. The liver architecture of mice injected with AdIP1 was seriously damaged with a massive infiltration of inflammatory cells (pink staining in H&E; Fig. 3). In these conditions, very significant staining for E1A was detected at 48 hours and increased by 5 days (Fig. 3). By contrast, H&E staining revealed a normal liver architecture and very little expression of E1A for all animals injected with AdIP2 although with evidence of inflammatory infiltrates (Fig. 3). These results show that E1A expression is tightly controlled in AdIP2.

**In vivo imaging of the propagation of AdIP2 in tumors.** HCT116 s.c. tumors were injected with 10^9 pfu of AdIP2. The animals were scanned with a small animal SPECT/CT scanner upon injection of ^99m^TcO_4^- . Figure 4A shows that ^99m^TcO_4^- accumulation is detectable 24 hours after viral injection and reaches a peak at 48 hours. From day 3, no ^99m^TcO_4^- accumulation was observed. Quantitative analysis of the images confirmed that the peak of hNIS-mediated accumulation of iodide was observed 48 hours after administration of the virus (Supplementary Fig. S5). Sections on tumor biopsies were prepared and stained with H&E (Fig. 4B). E1A immunohistochemistry was maximal 24 hours after injection of the virus and declined afterward. By day 5, E1A expression was only detectable in necrotic area of the tumor (Fig. 4C). Immunohistochemical analysis of hNIS expression revealed that a hNIS-specific signal could be detected at all times (Fig. 4D). However, at days 1 and 2, the hNIS-expressing cells seem intact, and hNIS immunoreactivity is located at the plasma membrane of the cancer cells. By contrast, from day 3, hNIS staining is clearly diffuse and associated with areas of cell death and necrosis. These data suggest that hNIS immunoreactivity remains present in the tumor, but the lack of signal in SPECT imaging is likely to be due to the fact that the protein is not present in live healthy cells capable of concentration of ^99m^TcO_4^- .

**Retention of iodide at the peak of hNIS expression.** HCT116 s.c. tumors were injected with 10^9 pfu of AdIP2. Forty-eight hours later, the animals were injected with a tracer dose of Na^{125}I.
A different time points, animals in the cohort (n = 4) were culled; the tumors, as well as plasma samples, were collected, and the radioactivity associated was counted. The tumor/plasma ratio of the specific activity was 3.0±0.2 30 minutes after radioiodide injection, 3.4±0.3 after 1 hour, 2.75±0.4 after 4 hours, 1.5 ± 0.5 after 10 hours, and 1.0±0.2 after 14 hours. These data confirm that iodide is accumulated in the tumor. However, the retention is limited as the iodine concentration in the tumor is equivalent to that in the plasma 14 hours after radioisotope administration.

**Therapeutic effect** in vivo. We next compared the therapeutic efficacy of AdIP2 (Wnt selective; hNIS positive) and AdIP4 (Wnt selective; hNIS negative) *in vivo*. Mice bearing HCT116 tumors were treated with a single intratumoral injection of the viruses (10⁹ pfu) or control. Figure 5 shows that both viruses were capable of reducing tumor progression compared with PBS-injected animals (for both viruses, P < 0.001). In contrast to the situation *in vitro* (Supplementary Figs. S1-S4), no statistically significant differences were observed between the therapeutic efficacy of AdIP2 and AdIP4 (Fig. 5). Injection of therapeutic doses of ¹³¹I in conjunction with the oncolytic viruses was also tested. ¹³¹I administration was done 48 hours after infection. Figure 5 shows that a single dose of ¹³¹I combined with a single injection of AdIP2 is capable of inducing tumor regression and that this additional therapeutic effect is absent when the hNIS-negative AdIP4 is combined with ¹³¹I (P < 0.001 for AdIP2 versus AdIP4; in the presence of ¹³¹I).

**Fig. 4.** Kinetics of spread of AdIP2 *in vivo*. Balb-c nude mice were seeded subcutaneously with HCT116 cells (2 × 10⁶). When the tumors reached around 1 cm² in size, they were injected with 10⁹ PFU of AdIP2. Mice (n = 10) were scanned using a dedicated small animal SPECT/CT scanner and representative scans showing ¹⁹⁹mTcO₄⁻ accumulation in the tumor are shown (A). Every day, two animals were culled and the tumor processed for H&E staining (B) and E1A or hNIS immunohistochemistry (C) or (D) (x10), respectively.

**Fig. 5.** Comparison of the viro-radiotherapy induced by AdIP2 and AdIP4. Balb-c nude mice were seeded subcutaneously with HCT116 cells (2 × 10⁶). When the tumors reached around 1 cm² in size, they were injected with 10⁹ PFU of AdIP2 or AdIP4. Forty-eight hours later a single dose of ¹³¹I (1.5 mCi) was administered intra-peritoneally. The data represent means ± SEM of the tumor measurement, with 6 animals per experimental group. Two-way ANOVA statistical analysis was performed. These results are representative of two experiments.
Discussion

Comparison of oncolytic adenoviruses encoding the hNIS with their hNIS-negative counterparts showed that hNIS had a detrimental effect on the cytotoxicity and replication of the adenovector in vitro. Because the expression of hNIS from a replication-incompetent adenovirus was not associated with a particular side effect on a whole range of carcinoma cell lines (31–34), an explanation for this loss of potency of the viruses may be the increased size of the adenoviral genome containing the hNIS compared with its gp19k-deleted or wild-type counterparts. The relationship between adenoviral genome size and viral yield has already been studied in great details (35). The hNIS cDNA is around 2 kb in size, and this extra burden of viral DNA to replicate and package may affect the yield of viral progeny.

In vivo imaging of the spread of AdIP2 in the tumor shows that hNIS expression reaches a peak 48 hours after intratumoral injection, followed by a sharp disappearance of hNIS-dependent accumulation of radiotracer. This kinetic is identical to that obtained with AdIP1 (5) and suggests that oncolytic adenoviruses have a very limited propagation in tumors. Immunohistochemical analysis of hNIS expression revealed that hNIS immunoreactivity can be detected even at time points when no accumulation of radiotracer was detectable by SPECT (Fig. 4). However, hNIS was expressed in cells that seemed necrotic (Fig. 4D) and, therefore, incapable of concentration and retention of $^{99m}$TcO$_4^-$.

The visualization of the presence and spread of oncolytic adenoviruses has also been monitored by bioluminescence or fluorescence imaging in the past (36–39). In most studies, the presence of the reporter gene/protein decreases rapidly, confirming the very transient nature of transgene expression in oncolytic adenoviruses. In one study, the peak of expression was reached 4 days after intratumoral injection and was detectable after day 12 (36). The reporter gene used in that study was the enhanced green fluorescent protein, and as in our work, this reporter cassette was inserted in the E3 region. In the case of enhanced green fluorescent protein, it is likely that this protein will continue to emit fluorescence upon stimulation even after release by dead cells. In the same context, hNIS is likely to be nonfunctional and therefore undetectable by SPECT. These observations highlight the differences in signals obtained with reporter systems that do not require cell viability to be detectable.

In terms of therapeutic effect in vivo, no statistically significant difference was observed between AdIP2 and AdIP4 (Fig. 5). This is in sharp contrast with the situation in vitro, in which AdIP4 was five times more potent than AdIP2 (Supplementary Fig S3; in HCT 116 cells, EC50 for AdIP2 is 4.8 against 0.8 pfu for AdIP4). In addition, AdIP4 was shown to be able to release >10 times more viral progeny than AdIP2 upon infection of HCT116 cells (Fig. 2). These data, combined with E1A immunohistochemistry (Fig. 4C), suggest that in vivo adenoviral oncolysis represent a “one shot” strategy in which the therapeutic effect is achieved rapidly after virus inoculation, as opposed to delayed and mediated by autoamplification of the virus.

In the case of hNIS-mediated radiotherapy, the information gathered through SPECT imaging allowed a precise determination of the optimal timing for administration of the therapeutic dose of $^{131}$I. A single injection of a therapeutic dose of $^{131}$I, following a single injection of $10^9$ pfu 48 hours earlier resulted in a very significant decrease in tumor size, which was not obtained with the hNIS-negative AdIP4. These data show for the first time that hNIS-mediated viroradiotherapy is relevant to oncolytic adenoviruses and that NIS-mediated imaging can be used to individualize a treatment.

According to the International Commission on Radiation Protection, the dose absorbed by the bone marrow per unit of activity is 0.035 mGy/MBq for $^{131}$I (iodide; adult human with a blocked thyroid). The commonly admitted upper limit for a tolerable dose absorbed by the bone marrow is 2 Gy (40). This figure would allow 57 GBq of $^{131}$I iodide to be administered to a human adult with a blocked thyroid. A commonly used manner to extrapolate the injected activity between species is simply to scale by body weight. In this context, 1.5 mCi (55 MBq) injected to a mouse with a whole body weight of 25 g would correspond to 155 GBq administered to an “average” 70-kg human. We can therefore estimate that the dose of 1.5 mCi administered to mice must be decreased by at least a factor 3 for this approach to be translatable to humans.

This requirement for high doses of $^{131}$I is due to the relatively short-term retention of $^{131}$I in the tumor, and various strategies have been proposed to improve this retention time: Attempts to “organify” iodide in tumors have been made by expressing the thyroperoxidase cDNA (41, 42). However, the coexpression of NIS and thyroperoxidase did not increase significantly this retention time (42). More recently, a pharmacologic approach has been suggested with the use of a chemical compound capable of increasing iodide retention in vivo (43). However, the properties of this compound remain to be validated in vivo. The anatomic localization of the tumor seems to be very important for the retention of iodine. Unusually long-term retention of iodine has been reported in in situ tumors of the liver expressing hNIS (15). Finally, the combination of oncolytic viruses and metabolic radiotherapy with radiosensitizers could improve the therapeutic efficacy at a reduced dose of $^{131}$I. Therefore, the combination of oncolytic adenovirus encoding hNIS and pharmacologic radiosensitizers in the context of in situ liver tumors could turn this experimental approach into a strategy transferable to patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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