Adenovirus-mediated Transfer of the Thyroid Sodium/Iodide Symporter Gene into Tumors for a Targeted Radiotherapy

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ABSTRACT

The Na\textsuperscript{+}/\textsuperscript{I}\textsuperscript{–} symporter (NIS) present in the membranes of thyroid cells is responsible for the capacity of the thyroid to concentrate iodide. This allows treatment of thyroid cancers with \textsuperscript{131}I. We propose to enlarge this therapeutic strategy to nonthyroid tumors by using an adenoviral vector to deliver the NIS gene into the tumor cells. We constructed a recombinant adenovirus encoding the rat NIS gene under the control of the cytomegalovirus promoter (AdNIS). Infection of SiHa cells (human cervix tumor cells) with AdNIS resulted in perchlorate-sensitive \textsuperscript{125}I uptake by these cells to a level 125–225 times higher than that in noninfected cells. Similar results were obtained for other human tumor cell lines, including MCF7 and T-47D (mammary gland), DU 145 and PC-3 (prostate), A549 (lung), and HT-29 (colon), demonstrating that the AdNIS vector can function in tumor cells of various origins. In addition, AdNIS-infected tumor cells were selectively killed by exposure to \textsuperscript{131}I, as revealed by clonogenic assays. To assess the efficiency of this cancer gene therapy strategy in vivo, we injected the AdNIS vector in human tumors (SiHa or MCF7 cells) established s.c. in nude mice. Immunohistological analysis confirmed the expression of the NIS protein in the tumor. Three days after intratumoral injection, AdNIS-treated tumors could specifically accumulate \textsuperscript{125}I or \textsuperscript{123}I, as revealed by kinetics and imaging experiments. A quantitative analysis demonstrated that the uptake in AdNIS-infected tumors was 4–25 times higher than that in nontreated tumors. On average, 11% of the total amount of injected \textsuperscript{125}I could be recovered per gram of AdNIS-treated tumor tissue. Altogether, these data indicate that AdNIS is very efficient in triggering significant iodide uptake by a tumor, outlining the potential of this novel cancer gene therapy approach for a targeted radiotherapy.

INTRODUCTION

Iodide accumulation in the thyroid can reach concentrations 20–40-fold over the plasma levels (1). Iodide captured by the thyroid is organified, i.e., oxidized into iodine and subsequently used to iodinate thyroglobulin, the precursor of thyroid hormones. This unique capacity to concentrate and organify iodide allows the use of radioactive iodine isotopes (\textsuperscript{131}I) for the treatment of differentiated thyroid cancers and hyperthyroidism (2, 3). However, iodine metabolism abnormalities such as defects in iodide uptake and/or organification are frequently observed in thyroid cancer tissues, thereby seriously compromising the efficiency of radioiodine treatment (4–6).

Iodide accumulation in the thyroid gland is ensured by the NIS, a transmembrane glycoprotein present in the basolateral pole of thyroid follicular cells. NIS-mediated iodide uptake is an active transport process that occurs against the electrochemical gradient in \textsuperscript{–}anions and is competitively inhibited by thiocyanate and perchlorate anions (7, 8). The cDNAs of the rat and human NIS genes have been cloned recently and code for proteins of 618 and 643 amino acids, respectively (9, 10). The two proteins are 84% identical and have been predicted to be integral membrane proteins displaying 12 (9–11) or 13 membrane-spanning domains (12). NIS expression is not strictly limited to the thyroid but also occurs in several extrathyroidal tissues, including the salivary glands, the gastric mucosa, and the mammary gland (13). In these tissues, however, iodide is not organified (13).

The cloning of the NIS gene constitutes an important step toward the understanding of the molecular mechanisms underlying iodide transport abnormalities in thyroid pathologies. Indeed, several cases of hypothyroidism with low iodide uptake were linked to inactivating mutations in the NIS gene (14, 15). Similarly, the low or absent iodide transport observed in thyroid cancer tissues was correlated with a low or absent NIS expression (6, 16, 17). Importantly, NIS-mediated iodide transport does not require the follicular organization of the thyroid, as demonstrated in vitro in bovine (18), porcine (19), human (20), and rat thyroid cells (7). Several in vitro studies also showed that transfer of the NIS gene into nonthyroid cells, either by transfection of NIS cDNA (9, 10, 21, 22) or with a retroviral vector (23), led to iodide uptake by the transduced cells. Coupling delivery of the NIS gene into tumor cells with \textsuperscript{131}I administration may therefore open new avenues to treat cancer.

Adenoviral vectors are particularly well suited for cancer gene therapy. They lead to a transient but robust expression of the transgene, and efficient in vitro gene transfer has been reported in numerous tissues, including the thyroid (24–26). In addition, concentrated adenovirus preparations can be obtained, which constitutes a clear advantage over other viral vectors such as retroviruses for an optimal in vivo gene transfer. The commonly used adenoviral vectors are extremely attenuated and lack at least the viral early transcription regions E1 (essential for replication) and E3. Such vectors still exhibit some level of cytotoxicity, which can be viewed as an advantage for destructive strategies such as cancer gene therapy (for review, see Ref. 27).

The aim of this work is to demonstrate the feasibility of using an adenoviral vector to deliver the NIS gene into human tumors, with the goal of enabling them to concentrate radioactive iodine. For this purpose, we constructed and characterized a recombinant adenovirus expressing the rat NIS gene (AdNIS) as a first step toward a targeted radiotherapy of tumors.

MATERIALS AND METHODS

Construction of the Recombinant AdNIS Virus. AdNIS is a \textsuperscript{Δ}E1–ΔE3 recombinant adenovirus expressing the rat NIS gene under the control of the immediate early promoter of the cytomegalovirus (pCMV). The rat NIS gene [nucleotides 74–2046 of the published sequence (9)] was cloned as a SalI (blunt-ended by Mung Bean nuclease treatment)-HindIII fragment in the PvuII-HindIII sites of the pCEP-4 vector (Invitrogen) to add a promoter
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(pCMV) and a polya (SV40 polya). The obtained plasmid was called pAB1. A 3711-bp SstI-EcoRV fragment of pAB1 was then cloned in the shuttle vector pXL3048 linearized by EcoRV. pXL3048 is a KmR-SacB-ColEI derivative (28) containing the left end of the Ad5 genome (nucleotides 1–386), a polylinker with three unique cloning sites (EcoRV, BamHI, and SalI), and part of the Ad5 pIX gene (nucleotides 3446–4296). The shuttle vector containing the NIS expression cassette was called pAB2. The recombinant adenoviral genome encoding NIS was obtained by homologous recombination between plasmids pAB2 and pXL3215 in Escherichia coli, as described previously (28). pXL3215 contains the Ad5 genome bordered by two PacI sites and carrying deletions within E1 (nucleotides 386–3446) and E3 (nucleotides 28992–30470). After recombinational cloning in E. coli, the adenoviral genome was excised by PacI digestion, and the AdNIS virus was recovered by transferring the ligated and digested DNA into 293 cells by the Lipofectamine-based procedure (Life Technologies, Inc.).

Control Adenoviral Vectors. Recombinant adenoviruses expressing no transgene (AdCO1) or encoding β-galactosidase (AdβGal) were used as negative controls in this study and have been described previously (29, 30).

Virus Amplification, Purification, and Titration. All viral stocks were prepared from infected 293 cells (31) by standard procedures (30). After a two-step purification on CsCl gradients, viral stocks were desalted by using Pharmacia G50 columns (Orsay, France) and frozen at −80°C in PBS containing 7% glycerol. Viral titers were calculated by dilution plaquing onto 911 cells (32) and expressed in PFU/ml.

Cell Lines. Unless stated otherwise, all cell culture media and reagents were purchased from Life Technologies, Inc. Rat thyroid FRTL-5 cells (ATCC CRL-2409) were routinely grown in Coon’s modified Ham’s F12 medium (Sigma) supplemented with 5% donor calf serum (Life Technologies, Inc. 16030) and 10 μg/ml insulin (Sigma I 1882), 10 nm hydrocortisone (Sigma H 0396), 5 μg/ml transferrin (Sigma T 1147), 10 ng/ml somatostatin (Sigma S 1763), 10 ng/ml glycyld-L-histidyl-L-lysine acetate (Sigma G 7387), and 10 millimeters/ml thyrrotropin (Sigma T 8931). SiHa (ATCC HTB-35), MCF7 (ATCC HTB-22), T-47D (ATCC HTB-133), DU 145 (ATCC HTB-81), and SiHa (ATCC HTB-133), DU 145 (ATCC HTB-81), and HT-29 (ATCC HTB-38) cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, PC-3 cells (ATCC CRL-1435) were grown in F-12K Nutrient Mixture (Kaihn’s modification) supplemented with 10% heat-inactivated FBS. A549 cells (ATCC CCL-185) were maintained in minimum Eagle’s medium supplemented with 10% heat-inactivated FBS and 1% nonessential amino acids. 293 and 911 cells were grown in minimum Eagle’s medium supplemented with 10% FBS and 1% nonessential amino acids and DMEM supplemented with 10% FBS, respectively.

Anti-NIS Antibodies. A peptide spanning the COOH-terminal (600 – 618) region of NIS was synthesized with a conventional solid-phase method using an Applied Biosystems Model 431A peptide synthesizer. The identity and purity of the peptide (600–618) peptide were verified by amino acid analysis and peptide microsequencing. The synthetic peptide was conjugated to keyhole limpet hemocyanin using benzidine as the coupling agent on the Lysε residue. Two rabbits were immunized by intradermal injection of the synthetic peptide-carrier conjugate. After two boosts at 3-week intervals, animals were bled, and their sera were tested in an ELISA. Anti-NIS antibodies were purified on a nickel affinity column and used in the ELISA to detect the presence of the NIS immunogen. Cell-bound NIS was detected by an indirect immunofluorescence assay using a rabbit polyclonal IgG anti-NIS antibody (Vector Laboratories, Inc., Burlingame, CA). After further washing in PBS-0.5% FBS, slides were mounted and observed with a fluorescence microscope.

Infection Conditions for Iodide Uptake Experiments. Cells were seeded in 24-well plates 3 days before the experiment to achieve between 5 × 10³ and 10⁶ cells/well at the day of infection. Cell numbers were determined immediately before infection as the average cell content of two wells. Cells were infected at the indicated MOI in 200 μl of medium for 1 h, and then 800 μl of medium were added in each well. For each cell line, the medium used for infection was the same as the culture medium.

In Vitro ¹²³I Uptake Experiments. Iodide uptake experiments were performed 28–30 h after virus infection, using the method of Weiss et al. (7). Briefly, cells were washed once with 1 ml of HBSS buffered to pH 7.5 with 10 mM HEPES (bHBSS). Iodide uptake was then initiated by adding 0.5 ml of bHBSS containing 0.1 μCi of ¹²³I per well. After the indicated time of contact with iodide, cells were washed once with ice-cold bHBSS and incubated for 20 min in 1 ml of ice-cold ethanol. The ethanol was then recovered, and radioactivity was quantified (cpm) with a well gamma-counter (Beckman gamma 5500 B).

In Vivo Cell Killing with ¹³¹I and Clonogenic Assay. Cells were seeded in 24-well plates and infected at a MOI of 10, as described above. Twenty-five h after infection, cells were washed once with 0.5 ml of bHBSS and incubated with 0.5 ml of bHBSS (control) or 0.5 ml of bHBSS containing 10 μCi of ¹³¹I. After 5 h of contact with ¹³¹I, cells were washed twice with bHBSS, trypsinized, and counted. For each condition [noninfected cells, AdNIS-infected cells, and cells infected with a control adenovirus (AdCO1)], cells were plated in triplicate in 6-well plates (1000 cells/well) and incubated for 1 week at 37°C. Cells were then washed once with PBS and stained with a cristal violet solution (250 ml, 0.5 g of cristal violet, 25 ml of 40% formaldehyde, 50 ml of ethanol, and 175 ml of water). Colonies of more than 30 cells were counted, and the means and SD were determined for each condition. Results are expressed as the percentage of surviving cells, i.e., the percentage of colonies obtained after treatment with ¹³¹I compared to treatment with bHBSS alone, and are representative of two separate experiments.

Tumor Induction and in Vivo Iodide Uptake Experiments. Female nude mice (6 – 8 weeks of age) were irradiated (5 Gy) the day before injection of the tumor cells. Tumors were induced by s.c. injection of 200 μl of sterile PBS containing 5 × 10⁶ SiHa or MCF7 cells. In the case of MCF7 tumors, the cell suspension contained 50% Matrigel (Becton Dickinson). When tumors had reached 5–8 mm in diameter (approximately 3 weeks after cell injection), a 10-day thyroxine treatment was initiated to suppress thyroid iodide uptake; each day, animals were injected i.p. with 2 μg of l-thyroxine (Roche) diluted in 100 μl of PBS. Seven days after the onset of the l-thyroxine treatment, the AdNIS virus was injected into the tumors (2 × 10⁵ PFU in 100 μl of PBS), and iodide uptake was assessed 3 days later. For kinetics and quantitative uptake experiments, 6 μCi of ¹²⁵I were injected i.p. in 200 μl of sterile PBS. The presence of radioactive iodide in the tumors was recorded up to 400 min after the injection of radioactive iodide, using a small radiation-sensitive probe (Europrobe-Eurorad, Strasbourg, France). For quantitative analysis of the amount of ¹²⁵I present in the tumors, mice were sacrificed 90 min after the injection of radioactive iodide, the weight of the tumors was determined, and radioactivity was quantified using a calibrated well gamma-counter (Compugamma 1282; LKB) for 1 min. For experiments with ¹³¹I, mice were injected i.p. with 30, 60, or 90 μCi of ¹³¹I in 200 μl of sterile PBS (corresponding in terms of radioactivity/weight to doses generally used in human therapeutic applications); five animals were included in each group, and tumor sizes were followed for 2 weeks. For imaging experiments, 50 μCi of ¹³¹I were injected i.p. in 200 μl of sterile PBS. 90 min later, animals were anesthetized, and an image was taken (gamma camera DHD, SMV, BUC France). In all cases, the tumors were removed and analyzed by immunohistology.

Immunohistology. Removed tumors were fixed in 5% acetic acid, 75% absolute ethyl alcohol, 2%, of formalin (40%), and 18% water for paraffin block preparations. To examine the histological aspect of the tumor, paraffin sections (5-μm thick) were stained with H&E-saffron. The presence of the NIS protein was revealed by incubating the sections for 1 h with anti-NIS polyclonal antibodies (see above) at a 1:300 dilution and then incubating sections for 30 min with a goat antirabbit secondary antibody conjugated to peroxidase (Envision; Dako). 3-Amino-9-ethylcarbazole (Envision kit; Dako) was used to reveal the markers, and sections were counterstained with Mayer’s hematoxylin (1:2).

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RESULTS

Construction and Characterization of the Recombinant Adeno-
virus Encoding the NIS Gene. We constructed an E1/E3-defective recombinant adenovirus encoding the NIS gene from rat thyroid FRTL-5 cells under the control of the immediate early promoter from the cytomegalovirus (AdNIS). To confirm expression of the NIS protein from the engineered adenovirus, we infected SiHa cells (human cervix tumor cells) with virus-containing culture supernatant. Twenty-four h after infection, cells were fixed and permeabilized. The presence of the NIS protein was then detected by immunofluorescence using a rabbit polyclonal antibody directed against the COOH-terminal part of the NIS protein and a FITC-labeled antirabbit antibody. Fig. 1A shows that AdNIS-infected cells were clearly labeled and that the NIS protein was localized at the cell surface. On the contrary, noninfected SiHa cells were not labeled (Fig. 1B). In addition, cells infected with a control adenovirus (Adβgal) and AdNIS-infected cells treated with PBS in place of the primary antibody did not display any staining (data not shown), confirming that the observed signal was linked to the expression of the NIS protein. The AdNIS vector thus leads to high-level expression and correct localization of the NIS protein within the infected cells.

Tumor Cells Infected with AdNIS Efficiently Accumulate Iodide. To further characterize the AdNIS virus, in vitro iodide uptake experiments were carried out on AdNIS-infected SiHa cells. Cells were infected with CsCl-purified AdNIS for 28–30 h at various MOIs. Iodide uptake was then measured by incubating the cells for 15 min with bHBSS containing 0.1 μCi of 125I. At a MOI of 10, cells accumulated 140 times more iodide than noninfected control cells (Fig. 2A). Surprisingly, at a higher MOI, a lower amount of iodide was taken up (Fig. 2A); possible explanations include a direct cytotoxicity of the adenoviral vector or a toxic effect of NIS overexpression in the cell membranes. In addition, cells infected with a control adenovirus (Adβgal) did not accumulate more iodide than noninfected cells (data not shown), demonstrating that iodide uptake was specifically linked

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to expression of the NIS protein. Taken together, these results demonstrate that the AdNIS virus drives expression of a functional protein in vitro and leads to efficient iodide uptake after infection.

**In Vitro Characterization of the Iodide Uptake Capacity of AdNIS-infected Cells.** NIS-mediated iodide uptake is specifically inhibited by perchlorate anions (9). We thus repeated iodide uptake experiments in the presence of various concentrations of NaClO₄. As a control, the experiment was done in parallel with FRTL-5 cells, which naturally express the NIS protein and concentrate iodide (7). Results are shown in Fig. 2, A and B. In FRTL-5 cells, iodide uptake was already inhibited more than 90% in the presence of 30 μM NaClO₄, as described previously (7, 23). Likewise, iodide uptake in cells infected by AdNIS (MOI 10) was inhibited by NaClO₄ in a dose-dependent manner. However, results were quite different from those obtained for the thyroid cells: at an inhibitor concentration of 30 μM, the process was only inhibited to 54%, and a concentration of 300 μM was required to inhibit uptake by more than 90%. A probable explanation for this observation is that SiHa cells are easily infected by adenoviruses and that AdNIS-infected cells thus probably express very high levels of NIS.

We then studied the kinetics of iodide uptake in AdNIS-infected SiHa cells and compared it with that of FRTL-5 cells (Fig. 2C). Equal numbers of FRTL-5 cells, noninfected SiHa cells, and AdNIS-infected SiHa cells (MOI 10) were incubated for 5–120 min with 0.1 μCi of ¹²³I, and the amount of incorporated iodide was quantified at several time points. As expected, noninfected SiHa cells did not accumulate iodide, even after a 2-h incubation with ¹²³I. Iodide uptake was very rapid in FRTL-5 cells, as described previously (7). In the case of AdNIS-infected cells, the initial kinetics of iodide uptake was similar to that observed for FRTL-5 cells, with a maximal level reached after 30 min. However, for longer incubation times, the amount of iodide retained in the cells decreased significantly, suggesting an efflux effect. Interestingly, AdNIS-infected cells accumulated three to five times more iodide than the FRTL-5 cells. Transfer of the NIS gene by the AdNIS vector thus leads to rapid and perchlorate-sensitive iodide uptake, as described for cells naturally expressing a functional NIS protein.

**The AdNIS Vector Is Functional in Various Human Tumor Cell Lines.** Iodide uptake experiments were performed on several human tumor cell lines, namely MCF7 and T-47D (mammary gland), PC-3 and DU 145 (prostate), A549 (lung), and HT-29 (colon) cells. For each cell line, MOIs of 10 and 25 were tested, and iodide uptake was measured after 15 min of contact with 0.1 μCi of ¹²³I. For all cell lines, AdNIS infection at a MOI of 10 led to a highly significant iodide uptake. The amount of iodide taken up varied slightly from one cell line to the other, being increased 35–225 fold as compared with noninfected cells (Fig. 3). These differences are partially due to small variations in cell numbers (see Fig. 3) but also reflect the capacity of the adenoviral vector to infect the different cell lines. Increasing the MOI did not have the same effect on all the cell lines tested. At a MOI of 25, MCF7, PC-3, A549, and HT-29 cells displayed an increased capacity to concentrate iodide, whereas almost no difference was observed for T-47D cells; on the contrary, the capacity of DU 145 cells to take up iodide decreased, similar to what we observed for
SiHa cells (see Figs. 2 and 3). These differences probably reflect the more or less pronounced cytotoxic effect of the adenoviral vector on the various cell lines tested.

To assess the specificity of the process, we also measured iodide uptake in each cell line in the presence of NaClO₄. As shown in Fig. 3, iodide uptake was again inhibited in a dose-dependent manner by perchlorate anions. However, in contrast to what was observed with AdNIS-infected SiHa cells, a concentration of 30 μM was sufficient to obtain an inhibition of more than 95%. This difference again reflects the capacity of the viral vector to infect the different cell lines, which is at least 10–20 times higher for SiHa cells than for the other cell lines.⁴ Taken together, these results demonstrate that AdNIS is functional in various tumor cell lines, whatever their origin.

**AdNIS-infected Human Tumor Cells Are Efficiently Killed by ¹³¹I.** In vitro ¹³¹I uptake experiments were performed on AdNIS-infected MCF7 and HT-29 cells to demonstrate that it was possible to obtain cell killing with the AdNIS-radioactive iodide system. Noninfected cells and cells infected with an adenoviral vector encoding no transgene (AdCO1) were treated similarly, as controls. After ¹³¹I treatment, clonogenic assays were performed, and results are shown in Fig. 4 and expressed as the percentage of surviving cells. In each case, for cells treated with bHBSS only, the numbers of colonies were comparable, indicating that infection by an adenovirus (AdNIS or AdCO1) did not affect cell survival. On exposure to ¹³¹I, around 30% of the cells were nonspecifically killed, as assessed by the results obtained for noninfected and AdCO1-infected cells. However, the number of colonies recovered from AdNIS-infected cells was significantly lower than that from noninfected or AdCO1-infected cells, showing a selective killing effect of ¹³¹I on NIS-expressing cells. These results thus demonstrate that coupling AdNIS and ¹³¹I treatments in vitro efficiently and specifically leads to cell killing, which is the end goal of the system.

**The AdNIS Vector Leads to Efficient Iodide Uptake in Vivo.** To evaluate the efficiency of the AdNIS vector in vivo, SiHa tumors were induced on the right flank of nude mice by s.c. injection of 5 × 10⁶ cells. The virus (2 × 10⁹ PFU) was injected directly into the tumors when they had reached a diameter of 5–8 mm, and ¹²⁵I uptake experiments were carried out 3 days after i.p. administration of 6 μCi of ¹²⁵I in NaClO₄.

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Fig. 3. The AdNIS vector leads to efficient iodide uptake in various cell lines. The iodide uptake capacity of MCF7 cells (mammary gland), DU 145 cells (prostate), A549 cells (lung), T-47D cells (mammary gland), PC-3 cells (prostate), and HT-29 cells (colon) was tested 29 h after infection with AdNIS at the indicated MOI. Iodide uptake was measured by incubation for 15 min with 0.1 μCi of ¹²⁵I in bHBSS in the absence or presence of NaClO₄ at the indicated concentration. Results are expressed in numbers of cpm and are representative of at least two separate experiments, each done in duplicate. Cells numbers per well were as follows: MCF7 cells, 7.8 × 10⁵; DU 145 cells, 6.5 × 10⁵; A549 cells, 5.5 × 10⁵; T-47D cells, 6.4 × 10⁵; PC-3 cells, 6.9 × 10⁵; HT-29 cells, 1 × 10⁶. NaClO₄, sodium perchlorate concentration (μM); −, no inhibitor.

⁴ A. Boland, unpublished observations.
To prevent $^{125}$I uptake by the thyroid gland, mice were treated with l-thyroxine. The amount of radioactivity present in the thyroid, in the tumor, and at a control point near the tumor was measured for 7 h by using a radiation-sensitive probe (Fig. 5A). As expected, a low amount of $^{125}$I was trapped by the thyroid gland. In untreated control SiHa tumors, there was no difference between the activity detected within the tumor or that detected in its vicinity. On the contrary, for mice harboring an AdNIS-treated tumor, the radioactivity detected within the tumor was significantly higher than that in the control region, demonstrating that AdNIS-treated tumors did specifically concentrate $^{125}$I. Similar results were obtained with MCF7 tumors, confirming that the AdNIS vector is functional in vivo (data not shown).

Unfortunately, endogenous NIS expression in the mouse stomach caused a high background level that was not avoidable with the radiation-sensitive probe, given the size of the animal. Thus, it was not possible to carry out a quantitative analysis of iodide uptake in the tumors by this method. To obtain more quantitative results, two tumors, one on each flank, were induced on 12 mice. One tumor was injected with AdNIS, and the other was used as a negative control. Ninety min after i.p. injection of 6 $\mu$Ci of $^{125}$I, mice were sacrificed, and the radioactivity present in the tumors was measured (Fig. 5B). In each mouse, the AdNIS-treated tumor accumulated significantly more radioactive iodide than the control tumor. Indeed, the amount of $^{125}$I present per milligram of tissue in the infected tumor was 4–25-fold higher than that observed for the control tumor (Fig. 5B; Table 1). On average, the radioactive uptake (percentage of the total amount of injected $^{125}$I) in the AdNIS-treated tumors was 11%/g tumor tissue (range, 4.3–16.8%/g tumor tissue; see Table 1 for details). To evaluate whether these radioactive iodide concentrations were sufficient to affect tumor viability and/or growth, we repeated the iodide uptake experiments as described above, but with $^{131}$I. Doses of 30, 60, or 90 $\mu$Ci of $^{131}$I were administered by i.p. injection to mice harboring an AdNIS-injected tumor, and tumor volumes were followed for 14 days. Unfortunately, no difference in tumor size could be observed between groups of animals treated with AdNIS, $^{131}$I and control groups treated with a control virus or with AdNIS alone (data not shown). In addition, histological analysis of all the tumors did not reveal any difference in terms of necrosis. This absence of therapeutic effect of $^{131}$I indicates that despite an efficient iodide uptake (see above), the radiation doses delivered in vivo to the tumors were not high enough to affect tumor cell survival. Similar observations have been made by Shimura et al. (33) working with transformed rat thyroid cells.

To visualize the NIS-expressing tumor in vivo, scintigraphy was carried out with $^{123}$I, an isotope that allows imaging with a conventional gamma camera. Fifty $\mu$Ci of $^{123}$I were injected i.p., and an image was taken after 90 min (Fig. 6). As expected, tissues naturally expressing NIS (stomach and thyroid) or involved in iodide elimination (bladder) were visualized. The AdNIS-infected tumor was clearly visible, with an intensity comparable to that of the thyroid or the bladder. On the contrary, a noninjected control tumor was not seen (data not shown).

**Immunohistological Analysis of Tumors Injected with AdNIS.** For all in vivo experiments, expression of the NIS protein in the tumor was studied by immunohistological analysis. Whereas no label was apparent in the control tumors (data not shown), all tumors injected with AdNIS were specifically labeled with the anti-NIS antibodies. The distribution of NIS-transduced cells within the tumor was heter-
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Table 1. Iodide concentrations in tumors after AdNIS treatment

Each mouse carried two tumors, one injected with 2 × 10^6 PFU of AdNIS and one untreated tumor used as a negative control. Ninety min after i.p. injection of 6 μCi of 125I, tumors were removed, their weight was determined, and the radioactivity present in each sample was counted. For each mouse, results were expressed as the percentage of the total amount of 125I injected that was recovered per gram of tumor tissue (radioactive concentration). The last column represents the AdNIS-treated tumor:control tumor ratio.

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ogogeneous, with foci of NIS-expressing cells representing up to 30% of the surface of the viable tumor tissue. Representative tumor sections are shown in Fig. 7. Interestingly, these foci were generally located at the border between necrotic and viable regions of the tumor (see Fig. 7, A–D). A possible explanation for this observation is that the adenoviral preparation injected could diffuse more easily at the contact of softer tissue (necrosis) and less so in viable areas. An alternative hypothesis is that necrosis could be linked to a direct cytotoxic effect of AdNIS infection. In some immunostained areas, it was clear that labeling was confined to the cell membranes, demonstrating that in vivo also, the AdNIS vector leads to correct localization of the NIS protein (Fig. 7, E and F).

DISCUSSION

The recent cloning of the NIS gene is not only of major importance for the understanding of the mechanisms underlying iodide transport in the thyroid but could also open new therapeutic perspectives. Indeed, achievement of efficient transfer of the NIS gene, coupled to radioactive iodide administration, may allow radiiodine treatment of nonthyroid tumors as well as thyroid tumors presenting a defect in their capacity to trap iodide.

In this study, we investigated the possibility of using an adenoviral vector to deliver the NIS gene into tumors, with the aim of treating them with radiiodine. We constructed a recombinant adenovirus, AdNIS, driving expression of the rat NIS gene under the control of the cytomegalovirus promoter. In vitro infection of human tumor cells from various origins, including mammary gland, prostate, lung, cervix, and colon, with AdNIS led to expression of a functional NIS protein, as revealed by efficient iodide uptake after infection. Iodide accumulation in AdNIS-infected cells was rapid and perchlorate sensitive, two characteristics previously reported for NIS-mediated iodide transport (9). Depending on the cell line tested, iodide accumulation was 35–225-fold higher than that in noninfected control cells. In addition, AdNIS-infected cells were efficiently killed by 131I, as revealed by clonogenic assays. The AdNIS vector was also functional in vivo, as revealed by the iodide accumulation observed in vivo in tumors injected with AdNIS. Taken together, our results demonstrate that using the AdNIS vector is a valid approach to achieve efficient iodide uptake in tumors of nonthyroid origin.

While this study was in progress, transfer of the NIS gene with a retroviral vector was described in human [A375 (melanoma) and IGROV (ovarian carcinoma)] and mouse [CT26 (colon carcinoma) and BNL.1 ME (transformed liver)] tumor cells (23). In the cell lines tested, iodide uptake reached to a maximum 21-fold that observed with nontransduced cells and 35-fold that of cells transduced with a control retroviral vector (23). Delivery of the NIS gene by an adenoviral vector is likely to be more efficient because the iodide uptake capacity of AdNIS-infected cells was increased up to 225-fold as compared with that of noninfected cells. Another major difference between both studies concerns the results obtained in vivo. Whereas Mandell et al. (23) induced tumors by injection of tumor cells first transduced in vitro with the NIS gene, we preferred to inject the AdNIS virus in established tumors, an approach required in a therapeutic situation. In that respect, adenoviral vectors present a clear advantage over retroviral vectors, namely, the ability to obtain concentrated preparations, which facilitates efficient in vivo gene delivery. Our results thus demonstrate for the first time that it is possible to transfer the NIS gene in a preformed tumor and to thereby confer a significant and relevant iodide uptake capacity in vivo.

The therapeutic efficacy of radiiodine is dependent on the radiation dose delivered to the target tissue (34). The delivered radiation dose is proportional to both the effective half-life of 131I in the tumor [combination of the physical (8.02 days) and biological half-lives of...
and the radioactive concentration in the tumor, which is the ratio between the total uptake of iodide and the tumor mass. The maximal amount of $^{131}\text{I}$ that can be given to a patient is limited by the radiation dose delivered to healthy tissues. To efficiently treat a tumor with $^{131}\text{I}$ after transfer of the NIS gene, it is therefore important to combine in the tumor a high uptake of iodide, which depends on the level of NIS expression, with a long retention time of iodide, which is linked to the organification of iodide into cell proteins.

Intratumoral injection of AdNIS leads to efficient iodide uptake, since uptake in treated tumors was up to 25-fold higher than that in control tumors (Fig. 5; Table 1). Moreover, an average radioactive concentration of 11% of the injected amount of $^{125}\text{I}$ per gram of tumor tissue was observed. This result is to be compared with iodide concentrations of about 1% per gram of tissue in normal human thyroid tissue and only 0.1% per gram of tissue or even less that are routinely observed in human thyroid cancer tissues (35). Importantly, such results were obtained even if NIS expression in the tumor was not uniformly distributed, with foci of transduced cells representing up to 30% of the tumor, whereas other areas were not transduced (see Fig. 7). This implies that the AdNIS vector could lead to even higher iodide accumulation levels if the proportion of cells expressing NIS after infection was increased. An interesting approach in that respect is the modification of the viral capsid to improve the capacity of the virus to infect the target cells (36–39). Higher transduction levels could also be achieved by injecting larger doses of the virus at various points in the tumor. This would also contribute to the homogeneity of NIS expression in the tumor, which is quite important to consider for a therapeutic effect. Indeed, although the β emission of $^{131}\text{I}$ can cover a short distance (maximum, 2–3 mm) in biological tissues and thus deposit energy into cells neighboring those expressing NIS, the radiation dose delivered decreases rapidly with the distance (35), and the bystander effect thus remains limited.

Another key issue is the retention of iodide in the target tissue (3). Indeed, although the AdNIS-$^{131}\text{I}$ system led to efficient cell killing in vitro (Fig. 4) and despite the high iodide concentrations reached in AdNIS-injected tumors in vivo (Fig. 5B; Table 1), $^{131}\text{I}$ administration
did not have any effect on tumor viability and growth in our study. This observation is most probably linked to the fact that the tumors did not retain iodide for a time period long enough to allow delivery of a radiation dose affecting cell viability. In vitro data confirmed that the tumor cells used in this study do not have the capacity to organize and thus retain the iodide taken up after AdNIS treatment (data not shown). This was similarly illustrated in a study of tumors consisting of malignantly transformed rat thyroid cells that had lost their capacity to concentrate iodide and that were transinfected in vitro with the rat NIS gene (33). Although the tumors efficiently concentrated iodide, no effect of 131I on tumor growth was observed because the rapid iodide efflux from the tumor did not allow the delivery of a radiation dose sufficient to inhibit cell growth (33). Several advances are conceivable to circumvent this lack of iodide retention in the tumors. First, the efficiency of NIS gene transfer–and thus the iodide uptake capacity of the target tissue—may be improved by the use of modified vectors and/or higher viral doses (see above). Second, the biological half-life of radioiodine in the tumor tissues could be increased by coupling transfer of the NIS gene with delivery of a gene involved in the iodide organization process, such as the thyroperoxidase (1).

In view of the obtained results, coupling transfer of the NIS gene by an adenoviral vector and radioiodine administration appears to be a very promising strategy for treating tumors of various origins. Although improvements to achieve higher radiation doses in the target tissue will be required, application of this “targeted radiotherapy” approach in patients will be facilitated by our long-standing experience with radioiodine for thyroid cancer therapy.

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REFERENCES

Adenovirus-mediated Transfer of the Thyroid Sodium/Iodide Symporter Gene into Tumors for a Targeted Radiotherapy

Anne Boland, Marcel Ricard, Paule Opolon, et al.