In vivo Radioiodide Imaging and Treatment of Breast Cancer Xenografts after MUC1-Driven Expression of the Sodium Iodide Symporter

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ABSTRACT

Purpose: Expression of the sodium iodide symporter (NIS) in the thyroid gland provides for effective imaging and treatment of thyroid cancer using radiolabeled iodide. Transfer of NIS into other tumors would expand the utility of this treatment to tumors of nonthyroid origin. MUC1 is a transmembrane glycoprotein that is overexpressed in many tumor types, including breast, pancreatic, and ovarian. The aim of this study was to create a construct containing NIS under the control of the MUC1 promoter to target expression specifically to MUC1-positive breast cancer cells.

Experimental Design: A replication-deficient adenoviral construct was created containing the MUC1 promoter followed by the human NIS gene. Iodide uptake assays, Western blot, and immunohistochemistry were used to confirm NIS expression and function. Breast cancer xenografts in mice were infected with Ad5/MUC1/NIS and then imaged and treated using radioiodide.

Results: A 58-fold increase in iodide uptake was observed in infected MUC1-positive T47D cells with no significant increase observed in MUC1-negative MDA-MB-231 cells or in cells infected with the control virus. The in vivo study yielded clear images of Ad/MUC1/NIS-infected tumor xenografts using 123I. Administration of a therapeutic dose of 131I resulted in an 83% reduction in tumor volume, whereas control tumors continued to increase in size (P<0.01).

Conclusions: These results show that the MUC1 promoter is capable of directing efficient and selective expression of the NIS gene in MUC1-positive breast tumor cells. This could potentially have applications for both imaging and therapy in a range of MUC1-positive tumor types.

INTRODUCTION

The sodium iodide symporter (NIS) is a transmembrane glycoprotein responsible for uptake of iodide into cells. The presence of NIS on the basolateral membrane of thyroid follicular cells has been exploited for many years with the use of radiolabeled iodide to treat thyroid disease. This noninvasive therapy has proven to be a safe and effective way to both image and treat thyroid cancer (1).

The cloning and characterization of NIS, along with the discovery of its presence in tissues other than the thyroid, has raised the possibility of using radioiodide for imaging and treatment of tumors of nonthyroid origin (2–5). This laboratory previously reported the use of the prostate-specific antigen (PSA; refs. 6, 7), cytomegalovirus (CMV; ref. 8), and probasin (ARRPB; ref. 9) promoters to drive NIS expression in prostate cancer cells and effectively treat prostate cancer xenografts in vivo with 131I. The current study was developed as a result of the success of the prostate cancer model, with the aim of extending this technique to the treatment of other tumor types, particularly breast cancer.

Iodide accumulation in breast tissue was first reported over 40 years ago (10) and was more recently shown to be mediated by mammary NIS (11) derived from the same cDNA sequence as that of thyroid NIS (12). NIS is primarily expressed in breast tissue during lactation when iodide is accumulated into breast milk and used by the neonate in the formation of thyroid hormones, which are essential for the development of the nervous system, skeletal muscle, and lung (1, 11, 13). Although NIS expression has recently been reported in breast cancer (11, 14–16), the amount of expression is variable and it seems unlikely that the level of functional, membrane-targeted expression would be sufficient to permit reliable imaging or treatment of most breast tumors. As a result of this, induction of NIS expression in breast cancer cell lines has been reported after stable plasmid transfection (17) and adenoviral infection using a nonspecific promoter (18, 19).

Transcriptional targeting of therapeutic genes reduces extratumoral toxicity and results in selective, tissue-specific expression of the required gene (20). The aim of the current study was to induce targeted, tumor-specific expression of NIS in breast cancer cells. The MUC1 promoter was chosen to drive NIS expression.

MUC1 is a transmembrane glycoprotein that is present on the surface of secretory cells as well as on a variety of hematopoietic cells (21). It is overexpressed in many tumor types including breast, pancreatic, lung, prostate, and ovarian (21–23). The incidence of MUC1 overexpression in breast and pancreatic tumors has been reported to be as high as 90% (24, 25) and...
recent reports have also shown MUC1 overexpression in hematologic malignant diseases, such as B-cell lymphoma and multiple myeloma (24–26). Elevated MUC1 expression is associated with increased metastatic potential and poor patient survival (21, 23). In fact, it has been reported that MUC1-positive tumors account for 72% of new cancers and 66% of patient deaths (27).

The MUC1 promoter has been successfully used in the past to target expression of therapeutic genes to tumor tissues that overexpress the protein (28–35). In the present study, we have achieved targeted expression of NIS in breast cancer cells lines that express high levels of MUC1, permitting effective tumor imaging and therapy in vivo. Because overexpression of MUC1 correlates with high metastatic potential and poor patient survival, the ability to target NIS expression to such tumors may be highly beneficial. MUC1 overexpression is not limited to breast cancer alone and so this may have applications for both imaging and treatment of a variety of tumor types.

MATERIALS AND METHODS

**Cell Culture.** All cell lines were previously in stock or were purchased from the American Type Culture Collection (Manassas, VA). The following cell lines were used in this study: T47D, MDA-MB-231 (breast cancer); OVCAR-3, SK-OV-3 (ovarian cancer); and Capan-2, Panc-1 (pancreatic cancer). All media were supplemented with 10% fetal bovine serum and penicillin G (2 units/mL)/streptomycin sulfate (100 mg/mL). The media used for each cell line was as follows: T47D, DMEM; MDA-MB-231, RPMI; MDA-MB-231: L-15; SK-OV-3, Capan-2: McCoy’s 5A; Panc-1: DMEM. Cells were maintained at 37°C, 5% CO2 with a media change twice weekly and passage every 7 days.

**Adenovirus Production.** A replication-deficient human recombinant adenovirus serotype 5 construct containing human NIS under the control of the MUC1 promoter (−1,401 to +33) was produced in collaboration with ViraQuest Inc. (North Liberty, IA) as previously described (9). Briefly, the MUC1/NIS insert was cloned into a VQ5k plasmid shuttle vector provided by ViraQuest and then transfected into 293 cells containing adenoviral DNA that had previously been restricted to remove a portion of the E1 region. Plaque lysates were then prepared and used to infect 100 mm plates of 293 cells. At complete cytopathic effect, cells were harvested, lysed, and functional analysis was done. After two more rounds of plaque purification, the recombinant adenovirus Ad/MUC1/NIS was expanded in 293 cells and purified by banding on CsCl density gradients followed by dialysis. Another replication-deficient adenovirus containing NIS under the control of the nonspecific CMV promoter was in stock from previous experiments (9) and had been created using the same technique.

**Adenoviral Infection of Cell Lines.** Cells were plated into 12-well plates the day before infection at a seeding density of 1.5 × 10^5 to 2.5 × 10^5 cells/well to reach ~60% confluence for infection. Cells were then washed and incubated in serum-free medium and the appropriate multiplicity of infection of virus added to each well. After a 3-hour incubation at 37°C, cells were washed and incubated in complete medium for 72 hours before analysis of NIS expression/function. All adenoviral infections were carried out at least in triplicate.

125I Uptake Studies. The ability of the cells to concentrate iodide was determined as described by Weiss et al. (36). Briefly after transfection, cells were incubated in HBSS containing 10 mmol/L HEPES, 10 μmol/L NaCl and 0.1 μCi Na125I/mL (pH 7.3), and 10 μmol/L KClO4, a competitive inhibitor of iodide uptake by NIS, was included in control wells. After a 45-min incubation at 37°C, cells were washed with ice-cold PBS, lysed with 1 mol/L NaOH, and concentrated iodide was measured on a γ counter.

**Immunohistochemical Analysis.** Cells were plated into two-chamber slides at the appropriate seeding densities before infection and immunohistochemistry. Slides were put on ice for 15 minutes and then cells were fixed in cold methanol at -20°C for 15 minutes. After methanol removal, cells were incubated in 10% normal goat serum diluted in PBS/0.05% Tween 20 for 30 minutes to block nonspecific binding. A mouse monoclonal antibody (1:2,000) directed against the human NIS protein (aa 468-643; ref. 37) was then applied for 60 minutes followed by washing in PBS/0.05% Tween 20. Cells were then incubated with a biotinylated goat–anti-mouse immunoglobulin (1:200) for 20 minutes. Cells were then washed and then peroxidase-conjugated streptavidin (1:300) was applied for 20 minutes. After washing, detection was carried out using a peroxidase substrate kit containing the chromogen diaminobenzidine (Vector Laboratories, Burlingame, CA). Cells were counterstained with malachite green for 5 minutes before mounting using Glycergel mounting medium (DAKO, Carpinteria, CA).

**Growth of Breast Cancer Xenografts in Mice.** Female athymic nude mice (Harlan Sprague-Dawley, Indianapolis, IN) were implanted with 60-day release estradiol pellets (Innovative Research of America, Sarasota, FL) to support breast tumor growth. Mice received a s.c. injection of 2 × 10^7 T47D cells suspended in 0.2 mL RPMI medium in one or both flanks. When tumors had reached an appropriate volume (≥100 mm^3), mice were placed on a low-iodine diet with T4-supplemented water (5 mg/L) to reduce thyroidal uptake of iodide. Following results of initial imaging studies, estradiol pellets were also removed at this point. All experiments were approved and carried out according to the guidelines of the Animal Care and Use Committee of Mayo Clinic (Rochester, MN).

**Adenoviral Infection of Tumor Xenografts.** Mice with tumors ≥100 mm^3 that had been maintained on the low-iodide diet were given intratumoral injections of 5 × 10^6 plaque-forming units of recombinant Ad5-MUC1-NIS or control virus in a total volume of 100 μL of PBS/0.3% sucrose/PBS. Tumors were injected at multiple sites to increase virus dispersal, using tuberculin syringes with 27-gauge needles.

**In vivo Tumor Imaging.** Three days after injection of virus into the T47D tumors, mice were given an i.p. injection of 0.5 mCi 131I and images were acquired on a γ camera with a low-energy, high-resolution collimator. Serial images were acquired at various time points from 1 to 48 hours after iodide administration. This permitted calculation of the biological half-life of the iodide within the tumor.

**131I Therapy.** Mice were grouped according to the size of the T47D tumors and given intratumoral injections of virus as described above. Three days after virus administration, mice were given an i.p. injection of either 3 mCi 131I or saline (control
The volume of tumors infected with Ad5-MUC1-NIS and control virus were monitored and compared on a weekly basis after administration of the iodide in both groups treated with $^{131}$I and those given saline. Statistical significance was determined using Student’s $t$ test.

RESULTS

Iodide Uptake in Ad5/MUC1/NIS-Infected Cells. Iodide uptake levels in cells were determined after infection with Ad/MUC1/NIS or control virus containing the nonspecific CMV promoter (Ad/CMV/NIS), or a promoter-less virus (Ad/NIS; Figs. 1 and 2). The level of iodide uptake in Ad/MUC1/NIS-infected T47D cells was essentially equal to that seen after infection with Ad/CMV/NIS at a multiplicity of infection (MOI) of 20 and 40 (Fig. 1). Iodide uptake began to plateau at a MOI above 80 of the Ad/MUC1/NIS virus although it continued to increase when NIS was under the control of the CMV promoter. There was an 18-fold increase in iodide uptake in infected cells compared with control, with optimal uptake observed 3 days after virus infection (results not shown). In the absence of a promoter, there was no increase in iodide uptake in the cells. There was no significant change in iodide uptake in the MUC1-negative MDA-MB-231 cells after infection with the Ad/MUC1/NIS construct (results not shown).

To determine specificity of the adenoviral construct for MUC1-positive cells, two ovarian (OVCAR-3, SK-OV-3) and two pancreatic (Capan-2, Panc-1) cancer cell lines were also infected with the construct (Fig. 2). One cell line from each tissue was MUC1-positive (OVCAR-3, Capan-2), whereas the others were essentially negative for the protein (SK-OV-3, Panc-1). Infection of the MUC1-positive cells resulted in an 8- to 15-fold increase in iodide uptake over control cells, whereas the MUC1-negative cells showed no ability to concentrate iodide. In all cases, iodide uptake could be blocked by inclusion of KClO$_4$, a known inhibitor of NIS function, in the incubation medium.

Immunohistochemical Staining of Ad5/MUC1/NIS-Infected Cells. To confirm correct membrane localization of NIS, cells were fixed in methanol after virus infection and immunostained using a highly specific mouse–anti-NIS antibody. Strong membrane-targeted immunoreactivity was detected in Ad/MUC1/NIS-infected T47D cells (Fig. 3B). Both the MUC1-negative MDA-MB-231 cells and those cells infected with the promoter-less virus (Ad/NIS) were negative for NIS immunoreactivity.

In vivo Tumor Imaging. Initial radiiodine imaging of tumor-bearing mice revealed a pattern of uptake correlating with the location of the multiple mammary glands in the mice, as well as at the site of the MUC1/NIS-infected tumor (Fig. 4A). The experiment was repeated in another group of mice in the absence of the estradiol pellet that had been used to support tumor growth (Fig. 4B). This revealed an image showing iodide uptake in the thyroid and stomach, which both express NIS, and in the bladder where iodide was being cleared in the urine. There was also a clear image of the tumor that had been infected with Ad/MUC1/NIS on the left flank, whereas the tumor infected with control virus on the right flank did not concentrate iodide (Fig. 4B). There was no detectable uptake of iodide in the mammary glands of this animal.

Multiple images were acquired from 1 to 48 hours after iodide administration to determine the rate of iodide efflux from the tumors. The average tumor activity per gram (corrected for acquisition time, background, and iodide decay) at 1, 3, 5, and
7 hours was 21%, 15%, 11%, and 7%, respectively, and had decreased to <2% by the 24-hour time point (Fig. 5). The biological half-life of the iodide in the tumor was calculated to be 5.8 hours.

**131I Therapy.** After intratumoral infection with adenovirus, mice were given an i.p. dose of either 131I or saline. Tumor volume \((L \times W \times H \times 0.51)\) was recorded weekly and compared among the following three groups: (a) Ad/MUC1/NIS + 131I, (b) Ad/MUC1/NIS + saline, and (c) Ad/NIS + 131I (Fig. 6). Within 1 week of iodide administration, there was a marked regression in the volume of tumors infected with Ad/MUC1/NIS. By week 10, these tumors were reduced to <17% of their original volume \((P < 0.01)\). Animals have been tracked up to 3 months from treatment with no regrowth of tumors observed. Tumors infected with control virus, or those infected with NIS followed by saline administration, retained their original volume or increased in size.

**DISCUSSION**

This study describes the use of the MUC1 promoter to drive NIS expression in breast tumor cells. MUC1 is overexpressed in many tumor types, particularly breast cancer, and this overexpression correlates with poor survival and increased metastatic potential (21). Because this protein is not solely overexpressed in breast cancer, it may also have potential for targeting NIS expression to other tumor types. Previous studies have, for example, described the use of the MUC1 promoter to target therapeutic genes to esophageal cancer (34), ovarian cancer (38), breast cancer (29, 32, 35), and multiple myeloma (33).

In the current study, we investigated the ability of breast cancer cell lines to take up iodide after transfection with an Ad5/MUC1/NIS construct. When calculated on the basis of iodide concentration per cell volume, infection of the MUC1-positive T47D cell line with Ad/MUC1/NIS resulted in a 58-fold increase of iodide uptake over control cells. Immunohistochemistry revealed strong membrane-bound immunoreactivity with minimal intracellular staining. The importance of correct posttranslational targeting of NIS to the cell membrane has been highlighted in many studies investigating impaired iodide uptake in thyroid carcinomas. This has lead to a number of investigations, yielding conflicting results, into NIS expression in thyroid cancer (37, 39--42). Therefore, iodide uptake by tumors does not seem to be because of NIS expression alone, but may involve a combination of factors including posttranslational modifications and membrane targeting. The efficient targeting of the protein to the membrane in these cells may be because of the fact that there is native NIS expression in some breast cancers and so the cells can provide the posttranslational modifications necessary for correct targeting of NIS to the cell membrane.

Cells that were infected with control virus (Ad/NIS) did not show any increase in iodide uptake or NIS expression over noninfected cells, demonstrating that NIS expression was specifically controlled by the MUC1 promoter. Also, in vitro specificity for MUC1-positive tumors was shown through the inability of the construct to induce iodide uptake in MUC1-negative cells while significantly increasing uptake in MUC1-positive breast, ovarian, and pancreatic cells. The required threshold level of MUC1 expression for this construct to be effective has not yet been established. The level of MUC1 expressed by the cell lines used in this study has been quantitated by other groups (43--45). Although the MUC1 expression level is of paramount importance, the level of NIS expression will also depend on the ability of the adenovirus to infect the cell [i.e., the level of Coxsackie adenovirus receptor (CAR) expression; ref. 46]. Further studies will be required to correlate MUC1, CAR, and NIS expression levels. Ongoing in vivo studies investigating the efficacy of this construct in ovarian and pancreatic tumors will provide further insight into the strength of this construct as a therapeutic agent in the treatment of tumors with varying MUC1/CAR levels.

The results presented herein show that the MUC1 promoter is capable of driving functional, membrane-targeted NIS expression. Although, as mentioned previously, there have been
other studies using the MUC1 promoter to target therapeutic genes to a variety of tumor types, NIS provides a significant advantage as it allows for noninvasive 123I imaging to confirm correct targeting of the gene before proceeding to 131I therapy. In vivo investigations were carried out to further investigate the potential of this construct for the imaging and treatment of breast cancer in mice. Initial imaging experiments revealed a pattern of iodide uptake correlating with the position of the multiple mammary glands in the mouse as well as at the site of the MUC1/NIS-infected tumor. B, image of a second mouse 10 days after removal of the estradiol pellet; this revealed an image showing iodide uptake in the thyroid and stomach, which both express NIS, and in the bladder, where iodide was being cleared in the urine. There was also a clear image of the tumor that had been infected with Ad/MUC1/NIS on the left flank, whereas the tumor infected with control virus on the right flank did not concentrate iodide. There was no detectable uptake of iodide in the mammary glands of this animal.

Fig. 4  In vivo T47D tumor imaging of two mice using a γ camera after administration of 0.5 mCi 123I. Three days before iodide administration, mice were given intratumoral injections of Ad/MUC1/NIS or control virus. A, image of a mouse with high serum estradiol levels as a result of an implant to support tumor growth; it revealed a pattern of uptake correlating with the location of the multiple mammary glands in the mice as well as at the site of the MUC1/NIS-infected tumor. B, image of a second mouse 10 days after removal of the estradiol pellet; this revealed an image showing iodide uptake in the thyroid and stomach, which both express NIS, and in the bladder, where iodide was being cleared in the urine. There was also a clear image of the tumor that had been infected with Ad/MUC1/NIS on the left flank, whereas the tumor infected with control virus on the right flank did not concentrate iodide. There was no detectable uptake of iodide in the mammary glands of this animal.

Fig. 5 123I retention in T47D breast tumor xenografts. Tumors were infected with Ad5/MUC1/NIS followed by an i.p. dose of 0.5 mCi 123I and images were acquired on a γ camera over a 48-hour time period. The percent of iodide accumulated at the tumor site was calculated and corrected for background, image acquisition time, and iodide decay. The average tumor activity per gram at 1, 3, 5, and 7 hours was 21%, 15%, 11%, and 7%, respectively, and had decreased to <2% by the 24-hour time point. The biological half-life of the iodide in the tumor was calculated to be 5.8 hours.

Fig. 6 In vivo radioiodine therapy of T47D breast tumor xenografts. Tumors were infected with Ad5/MUC1/NIS (solid line) or control virus (dashed line) followed by an i.p. dose of either 131I or saline. Within 1 week of iodide administration, there was a marked regression in the volume of tumors infected with Ad/MUC1/NIS followed by 131I therapy. By week 10, these tumors were reduced to <17% of their original volume (P < 0.01). Tumors infected with control virus or those infected with NIS followed by saline administration retained their original volume or increased in size.
MUC1-Driven NIS-Mediated Therapy for Breast Cancer

... to be under hormonal control in the mammary gland (11, 47, 48), with the greatest up-regulation seen in the presence of the pregnancy hormones prolactin, oxytocin, and estradiol (11). Although estradiol alone may not be optimal for induction of NIS expression, the high levels used here (>900 pg/mL) to support tumor growth may have been responsible for the up-regulation of NIS in the normal mammary tissue. Estradiol levels in patients would not be as high as those induced in the animal model and so no toxicity to normal breast tissue would be expected.

The radioiodide imaging experiment was repeated after removal of the estradiol pellet. In the absence of the high serum estradiol levels, there was no further uptake of iodide observed in the mammary gland, but iodide accumulation at the tumor site was clearly visible, with a calculated half-life of 5.8 hours. This highlights the main advantage of NIS as a therapeutic gene, the fact that imaging can be done to confirm correct protein localization before proceeding to therapy.

As well as at the site of the NIS-expressing tumor, iodide uptake was also observed in the thyroid, salivary glands, and gastric mucosa. Radioiodide therapy has an excellent safety profile with only minor side effects that may include nausea/vomiting and mild saliadenitis (49). In the thyroid, NIS expression is controlled by thyroid stimulating hormone, which can be suppressed by the use of T3 supplementation. A recent study investigating iodide uptake in breast cancer metastases reported a 75% reduction in thyroidal iodide uptake using T3 therapy (16). Because NIS is not regulated by thyroid stimulating hormone in any other tissue, this would not affect iodide uptake at the tumor site. Administration of a therapeutic dose of 131I resulted in a marked regression of tumors that had been infected with the MUC1/NIS construct. Within 3 weeks of iodide administration, there was a mean reduction of 50% of tumor volume, with tumors reduced to 17% of their original volume by week 10 (P < 0.01). Tumors infected with control virus, or those treated with saline after NIS expression, maintained their original volume or increased in size. The greatest increase in size was ~20% of starting volume, as growth rate would be expected to decrease after removal of the estradiol pellet. Studies have shown, however, that withdrawal of estradiol does not result in tumor regression (50) and only those tumors that had the combination of MUC1/NIS infection followed by 131I administration showed a reduction in volume. Ongoing in vivo studies will determine the efficacy of this construct in more actively growing tumors. These results are extremely promising, particularly because this construct is not breast tissue–specific and so may have potential applications in other MUC1-positive tumor types. Further investigations will be done to broaden the application of this therapy to other tumor types that overexpress MUC1.

REFERENCES


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