[CANCER RESEARCH 63, 1333–1338, March 15, 2003]

Radioiodide Treatment after Sodium Iodide Symporter Gene Transfer Is a Highly Effective Therapy in Neuroendocrine Tumor Cells[1]

Meike L. Schipper,[2] Alexander Weber, Martin Béhé, Rüdiger Göke, Werner Joba, Harald Schmidt, Till Bert, Babette Simon, Rudolf Arnold, Armin E. Heufelder, and Thomas M. Behr

Departments of Nuclear Medicine [M. L. S., A. W., M. B., T. M. B.], Gastroenterology, Endocrinology, and Metabolism [R. G., W. J., H. S., T. B., B. S., R. A., A. E. H.], and Clinical Research Unit for Gastrointestinal Endocrinology [R. G., H. S.], Philipps University Marburg, 35043 Marburg, Germany

ABSTRACT

This study evaluates the possibility of treating Bon1 and QGP pancreatic neuroendocrine tumor cells with radioactive iodide (^{131}I) after stable transfection with the thyroid sodium iodide symporter (NIS). NIS expression was driven either by the strong viral cytomegalovirus promoter or by the tissue-specific chromogranin A promoter. Using either approach, NIS expression was confirmed by reverse transcription-PCR and Western blotting. Uptake of radioactive iodide was increased ~20-fold by chromogranin A promoter-driven NIS expression and ~50-fold by cytomegalovirus promoter-driven NIS expression. Maximal uptake was reached within 15 min in QGP cells and 30 min in Bon1 cells. Effective half-life was 5 min in QGP and 30 min in Bon1 cells. No evidence of organification was detected by high-performance liquid chromatography and gel filtration chromatography. ^{131}I was a highly effective treatment in NIS-expressing QGP and Bon1 cells, reducing clone formation by 99.83 and 98.75%, respectively, in the *in vitro* clonogenic assay. In contrast, clone formation was not reduced in QGP and Bon1 cells without NIS expression after incubation with the same activity concentration of ^{131}I as compared with mock treated cells. Absorbed doses to QGP and Bon1 cells are up to 150 and 30 Gy, respectively. In addition, a direct cytotoxic effect of radioiodide was demonstrated in NIS-expressing Bon1 cells after ^{131}I incubation. In conclusion, radioiodide treatment after NIS gene transfer appears to be a promising novel approach in the therapy of neuroendocrine tumors if its highly encouraging *in vitro* effectiveness can be transferred to the *in vivo* situation.

INTRODUCTION

Gastrointestinal and pancreatic neuroendocrine tumors are rare tumors originating from neuroendocrine tissues (1). Clinical symptoms are often caused by the production of hormonally active substances by the tumor such as serotonin, gastrin, insulin, vasoactive interstinal peptide, pancreatic polypeptide, or substance P. Chromogranin A is produced by 80–100% of neuroendocrine tumors and serves as a reliable biochemical marker. The disease can be cured by early surgery, but the vast majority of tumors have metastases at the time of diagnosis, which makes palliation the cornerstone of management. Debulking surgery, liver artery embolization, and chemotherapy aim at tumor mass reduction, whereas somatostatin analogues and IFN are effectively used for control of symptoms (2, 3). Recently, radioactively labeled somatostatin analogues have been used in trials, with response rates ~30% (4). Response rates of cytoreductive approaches are generally below 60%, and long-term results are not convincing (5). New and more effective approaches are therefore needed in the treatment of neuroendocrine malignancies.

Iodide accumulating differentiated tumors of the thyroid have an excellent 5-year survival rate (90%; Ref. 6). Because of their ability to concentrate and organify radioactive iodide, tumor cells and metastases can be eliminated by radioiodide treatment after surgery. Indeed, radioiodide treatment is an independent prognostic factor for survival in patients with thyroid carcinoma, reducing the percentage of deaths caused by the disease by two-thirds in a recent analysis (6). This ability is conferred by expression of the thyroid NIS[3], a basolateral transmembrane protein that transports iodide and sodium into the thyroid/tumor cell along an electrochemical gradient maintained by the Na^+/K^+ ATPase. Recently, attempts have been made to transfer the ability to accumulate radioactive iodide to nonthyroid tumors. After transfection of the NIS gene into tumor cells of various origins, uptake of radioactive iodide was up to 225-fold of nontransfected controls (7–10). Clonogenic survival of NIS-expressing tumor cells was reduced by up to 75% compared with cells that did not express the NIS gene. Although most groups were unable to demonstrate a therapeutic effect *in vivo*, Spitzweg *et al.* (11) have presented highly encouraging *in vivo* data, reporting complete remissions in 60% of animals with NIS-expressing prostatic adenocarcinoma xenografts after treatment with 300 μCi of ^{131}I i.p.

The aim of this study was to investigate the possibility of radioiodide treatment after NIS gene transfer in pancreatic neuroendocrine tumor cells. Furthermore, the characteristics of iodide kinetics, presence or absence of organification, influence of radioiodide on clonogenicity, the correlation of absorbed dose and effect on clonogenicity, and the presence or absence of a direct cytotoxic effect were examined.

MATERIALS AND METHODS

Cell Culture. The human serotonin secreting pancreatic carcinoid cell line Bon 1 was cultured in DMEM/Nut mix F12 medium (Life Technologies, Inc., Karlsruhe, Germany) supplemented with 10% FCS (Life Technologies, Inc.), 100,000 UI/liter penicillin, and 100 mg/liter streptomycin. Human nonfunctioning pancreatic islet cell tumor line QGP was maintained in RPMI 1640 (PAN Biotech, Aidenbach, Germany) supplemented with 10% FCS, 100,000 UI/liter penicillin, and 100 mg/liter streptomycin. Cell lines were kept at 37°C in an atmosphere of room air with 5% CO_2.

Cloning of NIS Transfection Plasmids. NIS cDNA was amplified from a human thyroid cDNA bank. After the addition of a kozak sequence to the 5′ end to ensure efficient translation, it was cloned into the eukaryontic expression plasmid pcDNA3.1-V5-His (Invitrogen, Karlsruhe, Germany). The resulting plasmid was termed pcDNA3.1-CMV-NIS. As a negative control, the plasmid pcDNA3.1-(-)-NIS was constructed by removal of the CMV promoter from pcDNA3.1-CMV-NIS and religation. For tissue-specific gene expression, a 2300-bp fragment of the chromogranin A promoter described elsewhere[4] was inserted into pcDNA3.1-CMV-NIS after removal of the CMV promoter. This plasmid was named pcDNA3.1-CgA-NIS.

Generation of Stably Transfected Cell Lines. Cells were transfected by electroporation. QGP cells were transfected with pcDNA3.1-CMV-NIS and

Received 9/11/02; accepted 1/17/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported, in part by the Stiftung P. E. Kempkes (Kempkes Foundation), Marburg, Grant 12/2000 (to M. L. S.) and Deutsche Krebshilfe (German Cancer Aid) Grants 10-1600-Be2 (to T. M. B.) and 10-01193 (to A. E. H.).

[2] To whom requests for reprints should be addressed, at Klinik für Nuklearmedizin, Klinikum der Philipps-Universität Marburg, Baldinger Strasse, 35043 Marburg, Germany. Phone: 0049-6421-286-2815; Fax: 0049-6421-286-7025; E-mail: schipper@mailer.uni-marburg.de.

[3] The abbreviations used are: NIS, sodium iodide symporter; CMV, cytomegalovirus; RT-PCR, reverse transcription-PCR; HPLC, high-performance liquid chromatography; PI, propidium iodide.

[4] T. Bert, M. L. Schipper, and B. Simon. Cell specific expression and transcriptional regulation of the human chromogranin A promoter, submitted for publication.

pcDNA3.1-(-)-NIS, whereas Bon 1 cells were transfected with pcDNA3.1-CgA-NIS and pcDNA3.1-(-)-NIS. Stable clones were selected by addition of 500 μg/ml geneticin to the medium 2 days after transfection. The resulting cell lines were designated QGP+ (pcDNA3.1-CMV-NIS), QGP− (pcDNA3.1-(-)-NIS), Bon1C (pcDNA3.1-CgA-NIS), and Bon1− (pcDNA3.1-CMV-NIS).

Demonstration of NIS Expression in Stably Transfected Cells. To assess NIS transcription levels, RT-PCR was performed after isolation of total RNA from the cells using the RNeasy-kit (QIAgen, Hilden, Germany) with exon-bridging primers hNIS3 and 4 (5′-AACGAGGCTTCTTCTACACA; 5′-TTCAAGGGCTTTATTCCATCTCT). As an internal control, β-actin transcripts were amplified simultaneously using primers β-actin 1 and 2 (5′-TCATGTTTGAGACCTTCAA; 5′-GTCTTTGCGGATGTCCACG). To assess the level of NIS protein in the cell membrane, Western blotting was performed using membrane preparations of transfected cells. In brief, cells were homogenized in ice-cold homogenization buffer [5 mM Tris-Cl (pH 7.4), 300 mM sucrose, 0.1 mM EDTA, and 10 μM phenylmethylsulfonyl fluoride] and ultracentrifuged on a 41% sucrose gradient for 1 h at 4°C and 23,500 rpm. The membrane containing band was isolated, protein content determined according to Bradford, and stored at −80°C after dilution to the desired protein concentration with homogenization buffer. For Western blotting, 20 μg of membrane protein were separated on a 12% polyacrylamide gel, electroblotted to a nitrocellulose membrane (Macherey-Nagel, Düren, Germany), and blocked with Tris-buffered saline with 5% dry milk powder. As pcDNA3.1-V5-His contains a V5 antigen, which is attached to the COOH-terminus of the translation product, NIS can be detected by horseradish peroxidase-conjugated Anti-V5-Antibody (1:5000; Invitrogen) and visualized using enhanced chemiluminescence reagent (Amersham, Freiburg, Germany).

Uptake of ^{125}I by Stably Transfected Cells. Cells (4×10^4) were incubated for 1 h with 50 μCi/ml ^{125}I in 1 ml of HBSS, washed twice with ice-cold HBSS, and lysed with 0.1 M KOH. Radioactivity of lysates was determined using a Cobra II auto-gamma gamma counter (Packard BioScience, Dreieich, Germany). To assess internalization kinetics, incubation time with 50 μCi ^{125}I was varied to 5, 10, 15, 30, 45, 60, 90, and 120 min, respectively. For externalization studies, cells were incubated with 50 μCi ^{125}I for 1 h, washed twice with ice-cold HBSS, and incubated with nonradioactive HBSS for 5, 10, 15, 30, 45, 60, 90, and 120 min, respectively, before lysis. All experiments were performed in triplicate.

Assessment of Organification in Stably Transfected Cells. Cells (4×10^4) were incubated with 100 μCi/ml ^{131}I in 1 ml of HBSS for 12 h, washed twice with ice-cold HBSS, and lysed with distilled water. A total of 100 μl of the lysate was analyzed on a PD10 gel filtration column. Twenty fractions of 1 ml were eluted with 0.5 M sodium acetate (pH 5.4) and counted in a Cobra II auto-gamma gamma counter (Packard BioScience). A total of 50 μCi of ^{131}I in 100 μl of distilled water served as an internal control. For HPLC, proteins were denatured by addition of 500 μl of ethanol to 500 μl of lysate and centrifuged at 2500 g for 10 min. One hundred μl of supernatants were analyzed on a reversed phase C-18 HPLC column (Macherey-Nagel) in a HPLC 535 Detector (Biotek Instruments, Remsfeld, Germany) over an aqueous-acetonitril gradient. Radioactivity was detected continuously using a radiomatic flow scintillation analyzer (Packard BioScience). Again, 50 μCi of ^{131}I in 100 μl of distilled water were analyzed under the same conditions as an internal control.

***In Vitro* Clonogenic Assay.** Cells were incubated for 7 h in HBSS containing 100μCi/ml ^{131}I. After incubation, medium was changed several times over a period of 2 h to allow cells to externalize remaining ^{131}I. Cells were then seeded onto 6-well plates at densities of 200 cells/well in triplicate. After 2 weeks, colonies containing >50 cells were counted. The experiment was repeated with 50, 10, 5, 1, and 0 μCi/ml of ^{131}I, respectively. All experiments were performed in triplicate.

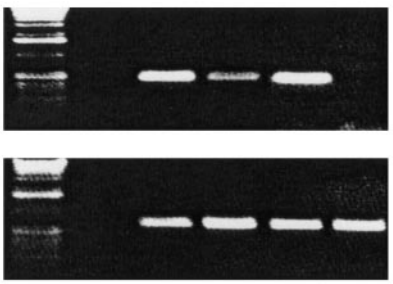

Fig. 1. RT-PCR of NIS-transfected cell lines with NIS (*top panel*) and β-actin (*bottom panel*) specific primers. Lanes from left to right are: marker; negative control; Bon1C; Bon1−; QGP+; and QGP−.

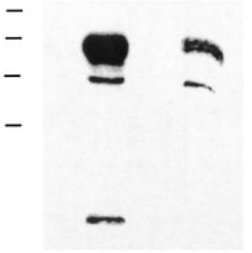

Fig. 2. Western blot of membrane preparations of NIS-transfected cell lines. Lanes from left to right are: indication of molecular mass (from top to bottom: 207, 120, 78, and 47 kDa), QGP−, QGP+, Bon1−, and Bon1C.

Dosimetry. To estimate the absorbed dose during the *in vitro* clonogenic assay, radioactivity measurements and kinetics from internalization and externalization experiments were extrapolated to the respective activity concentrations used in *in vitro* clonogenic assays. Counts/min were converted to disintegrations/min accounting for counter efficiency. Cumulative disintegrations/cell were determined from the area under the curve of a graph of disintegrations/min/cell *versus* time. Absorbed dose was calculated using S factors for 7-nm diameter cells from the literature (12). To correlate estimated dose and effect, clone formation was plotted against dose in a semilogarithmic plot. D_0, the dose required to reduce clone formation to 1/e (37%), was determined from the slope of a linear regression line fitted to the linear part of the curve.

Demonstration of a Cytotoxic Effect of ^{131}I. Cells were incubated for 7 h in HBSS containing 100 μCi/ml ^{131}I. After incubation, medium was changed several times over a period of 2 h to allow cells to externalize remaining ^{131}I. Cells were then seeded onto 6-well plates at densities of 10^6 cells/well in triplicate. Presence of cell death was assessed visually every day. Once dead cells appeared in the medium, cells were detached from plates, harvested, and resuspended in 200 μl of PBS. After staining with PI (final concentration 2.5 μg/ml), cells were analyzed by fluorescence aided cell sorting on a FACScan (Becton Dickinson, Heidelberg, Germany). All experiments were performed in triplicate.

RESULTS

Generation of NIS Expressing and Control Cell Lines. Three eukaryotic expression plasmids were constructed, expressing the NIS gene under the control of either the CMV promoter, the chromogranin A promoter, or without any promoter as a negative control. After transfection of human serotonin secreting pancreatic carcinoid cell line Bon 1 and human nonfunctioning pancreatic islet cell tumor line QGP with these plasmids, the following stably transfected cell lines were established by geneticin selection: Bon1C, expressing the NIS under the control of the chromogranin A promoter; QGP+, expressing the NIS under the control of the CMV promoter; and the negative controls Bon1− and QGP−, which contained the NIS plasmid lacking a promoter. RT-PCR revealed high levels of NIS gene transcription in Bon1C and QGP+ cells, with very low levels in Bon1− and no NIS gene transcription in QGP− cells (Fig. 1). Western blotting did not reveal any NIS protein in either QGP− or Bon1− cells. In contrast, we found strong bands of ~120 kDa, ~78 kDa, and ~15 kDa in QGP+ cells (Fig. 2). The 120-kDa and 78-kDa proteins were observed as considerably weaker bands in Bon1C cells as well.

NIS Is Functional in Transfected Neuroendocrine Tumor Cells. Both QGP+ and Bon1C cell lines accumulated high amounts of radioiodide, which was completely inhibited by addition of perchlorate (Fig. 3). Control cell lines QGP− and Bon1− did not concentrate

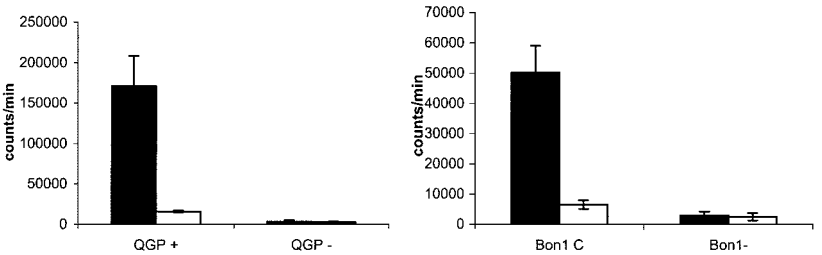

Fig. 3. Uptake of radioactive iodide by NIS-transfected QGP (*left panel*) and Bon1 (*right panel*) cells. Activity is shown without ■ and with perchlorate inhibition □.

radioactive iodide. Uptake of ^{125}I was 52-fold higher in QGP+ cells than in QGP− cells, and 18-fold higher in Bon1C cells compared with Bon1− cells. Accumulation was rapid in both QGP+ and Bon1C cells, reaching maximal levels within 30 and 15 min, respectively. Efflux of ^{125}I from QGP+ cells was rapid, with half maximal levels of activity reached after 5 min (Fig. 4). Interestingly, efflux from Bon1C cells was much slower than from QGP+ cells, resulting in an effective half life of 30 min for ^{125}I in Bon1C cells.

Iodide Is not Organified by NIS-transfected Neuroendocrine Tumor Cells. To assess whether iodide was organified in either QGP or Bon1 cells, we subjected lysates of cells that had been incubated for 12 h with ^{131}I to gel filtration chromatography and HPLC (Fig. 5). In gel filtration chromatography, activity was exclusively confined to the last nine fractions in all cell lines, and coeluted with free I^-. This indicates a small molecule such as free I^- and excludes organification into larger molecules such as proteins. In HPLC, lysates were analyzed on a C-18 column after denaturation of proteins to assess whether organification into smaller molecules such as lipids might play a role. In all cell lines, activity was exclusively confined to a single early peak at 3 min, indicating a small and highly polar molecule, which again coeluted with free I^-.

^{131}I Is a Strong Inhibitor of Clone Formation in NIS-expressing Neuroendocrine Tumor Cells. To evaluate the therapeutic potential of ^{131}I in NIS-expressing cell lines, *in vitro* clonogenic assays were performed using an activity concentration of 100 μCi/ml and HBSS only as a negative control (Fig. 6). Throughout all mock-treated cell lines, absolute numbers of colonies were approximately identical. For ease of comparison, numbers of colonies grown after treatment with ^{131}I are presented as percentage of numbers grown after mock treatment. Although numbers of colonies grown after radioiodide treatment of QGP− did not differ from mock-treated cells (100%), we observed a striking reduction in colony number in QGP+ cells. In most experiments, no clones at all were seen. Overall, colony formation was 0.17% of mock-treated controls. In Bon1− cells treated with radioiodide, a slight reduction in colony number was noted (88.1%). In contrast, in Bon1C cells, colony

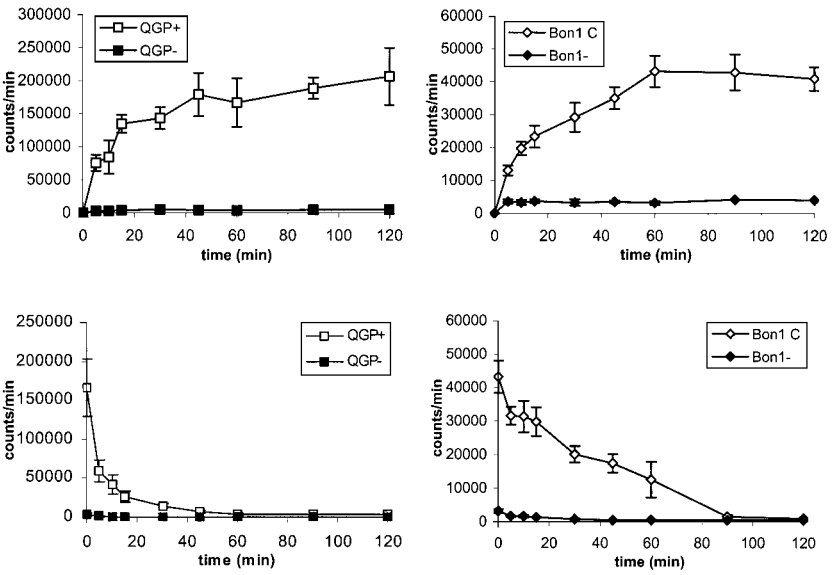

Fig. 4. Kinetics of radioiodide uptake in stably transfected Bon1 and QGP cells. Activity inside of cells is depicted over time. *Top left panel:* internalization of radioiodide into QGP+ and QGP− cells. *Top right panel:* internalization of radioiodide into Bon1C and Bon1− cells. *Bottom left panel:* externalization of radioiodide from QGP+ and QGP− cells. *Bottom right panel:* externalization of radioiodide from Bon1C and Bon1− cells. Results are expressed as mean and SD of at least three independent experiments.

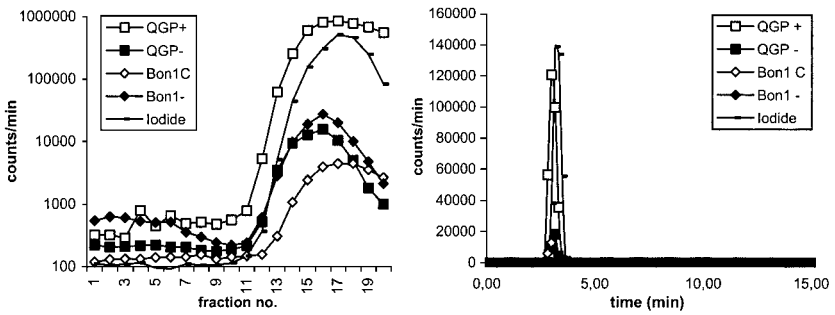

Fig. 5. Distribution of internalized activity in gel filtration chromatography (*left panel*) and HPLC (*right panel*). In the *left* panel, activity/eluted fraction is depicted for QGP+, QGP−, Bon1C, Bon1−, and free iodide. In the *right* panel, activity is measured continuously over time (min) during chromatography with an aqueous-acetonitril gradient in QGP+, QGP−, Bon1C, Bon1−, and free iodide.

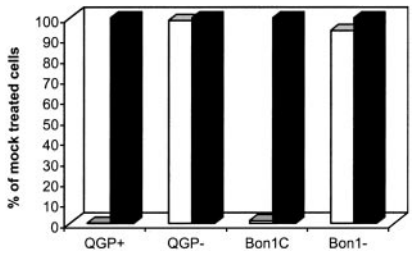

Fig. 6. Clone formation of NIS-transfected pancreatic carcinoid cells in the *in vitro* clonogenic assay after 7 h of incubation with 100 μCi ^{131}I/ml (□) or mock treatment (■). For ease of comparison, values are depicted as percent of mock-treated cells. From left to right: QGP+; QGP−; Bon1C; and Bon1−.

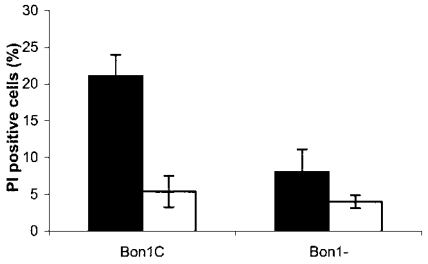

Fig. 7. Assessment of cell viability by PI exclusion of NIS-transfected Bon cells after 7 h of incubation with 100 μCi/ml ^{131}I (■) or mock treatment (□). Values are shown as percentage of PI-containing cells. From left to right: Bon1C treated with radioiodide/mock treated and Bon1− treated with radioiodide/mock treated. Results are expressed as mean and SD of at least three independent determinations.

formation was reduced to a degree similar to that seen in QGP+ cells (0.86%).

^{131}I Treatment Is Cytotoxic to NIS-expressing Neuroendocrine Tumor Cells. After incubation with 100 mCi/ml ^{131}I for 7 h, cells were assessed visually for the presence of a cytotoxic effect every day. When cells started to show signs of cytotoxicity after 5 days, PI exclusion was performed to quantify the portion of dead cells (Fig. 7). A total of 21.1% of Bon1C cells treated with radioactive iodide were unable to exclude PI, whereas only 5.4% of mock-treated Bon1C cells were. In contrast, only 8% of radioiodide-treated Bon1− cells could not exclude PI, which was not significantly higher than mock-treated Bon1C and slightly higher than mock-treated Bon1− cells (4%).

Dose-Effect Relationship of ^{131}I in NIS-transfected Cell Lines. The *in vitro* clonogenic assay was repeated using activity concentrations of 50, 10, 5, and 1 μCi/ml ^{131}I, respectively (Fig. 8). In both QGP+ and Bon1C cells, a nonlinear relationship was observed between activity concentration and reduction in clonogenicity. At 50 μCi/ml, clone formation was still markedly reduced in both QGP+ (4%) and Bon1C (13%) cells. In general, clone formation of QGP+ cells was inhibited more effectively at equal activity concentrations than of Bon1C cells (60 *versus* 79% at 10 μCi/ml and 63 *versus* 90% at 5 μCi/ml). At 1 μCi/ml ^{131}I, clone formation was unaffected (103 *versus* 99% for QGP+ and Bon1C, respectively).

Absorbed dose from internalized ^{131}I was calculated as described in the "Materials and Methods" section to correlate dose and effect of ^{131}I in NIS-transfected Bon1 and QGP cells. In Bon1 cells, absorbed doses ranged from 0 to 30 Gy. In QGP cells, absorbed doses were considerably higher, varying between 0 and 150 Gy. Clone formation was then plotted against calculated dose in a semilogarithmic plot (Fig. 9). High doses were required to effectively reduce clone formation in both neuroendocrine tumor cell lines. D_0, the dose required to reduce clone formation by 1/e, was 4 Gy for Bon1 and 13 Gy for QGP cells.

DISCUSSION

At present, there are no curative treatment options for metastatic neuroendocrine tumors. In contrast, thyroid cancer can be cured by radioiodide therapy even in advanced metastatic stages because of its ability to concentrate and organify radioactive iodide (13), which is conferred by the thyroid NIS. The cloning of the NIS gene has opened a new approach to the treatment of extrathyroidal malignancies by NIS gene transfer. Expression of functionally active NIS in a tumor would render it capable of accumulating radioactive iodide, thereby making it susceptible to radioiodide treatment (14).

Recently, there have been several successful attempts to induce iodide uptake by NIS gene transfer in various human cancer cell lines, including glioma, melanoma, liver, lung, colon, ovarian, cervix, prostate, mammary gland, and thyroid carcinoma cell lines (7–10, 15–18). As new strategies for the treatment of neuroendocrine tumors are needed, we have investigated the possibility of radioiodide treatment in neuroendocrine tumor cells after NIS gene transfer. We stably transfected the human serotonin secreting pancreatic carcinoid cell line Bon 1 and the human nonfunctioning pancreatic islet cell tumor line QGP with the NIS gene. Although the nonfunctioning cell line QGP is more typical of an *in vivo* nonfunctioning neuroendocrine pancreatic tumor, serotonin-secreting neuroendocrine tumors are more typically located in the midgut. However, both entities share the common feature of chromogranin A secretion, as do Bon 1 and QGP cells. NIS expression was driven by either the CMV promoter or the chromogranin A promoter to evaluate whether a tumor specific promoter such as the chromogranin A promoter would induce NIS expression and iodide uptake to a level sufficient for rendering pan-

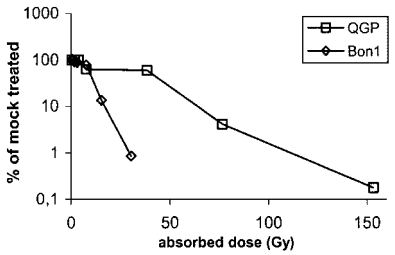

Fig. 9. Clone formation of NIS-transfected QGP+ and Bon1− cells in the *in vitro* clonogenic assay as a function of absorbed dose (Gy). Values are depicted as percent of mock-treated controls.

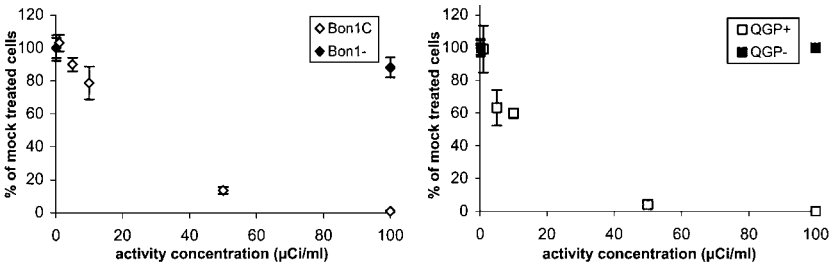

Fig. 8. Clone formation of NIS-transfected cells in the *in vitro* clonogenic assay as a function of the radioiodide concentration in μCi/ml. Values are depicted as percentage of mock-treated controls at concentrations ranging from 100 to 0 μCi/ml. *Left panel:* clone formation of Bon1C and Bon1−. *Right panel:* clone formation of QGP+ and QGP−. Results are expressed as mean and SD of at least three independent determinations. Some *SD bars* are hidden behind symbols.

creatic neuroendocrine tumor cells susceptible to radioiodide treatment. In addition, we sought to compare the level of NIS expression and the therapeutic potential of radioiodide of a tissue-specific promoter to that of a strong viral promoter.

Using either approach, we were able to demonstrate the induction of NIS mRNA and protein in Bon1C and QGP+ cells. Noteworthy, small amounts of NIS mRNA expression were observed in Bon1− cells. This is consistent with unpublished results by two of us (A. E. H., W. J.), who demonstrated NIS mRNA expression in wild-type Bon1 cells. However, no expression of NIS protein or uptake of ^{125}I was detected in Bon1− cells. NIS mRNA expression has been reported in several human tissues such as pancreas, adrenal, ovary, testes, or thymus, without proof of protein expression or iodide uptake (19–21). It is unclear whether NIS mRNA transcription without protein expression is of any physiological relevance. The unglycosylated protein backbone of NIS is seen as a ~65-kDa band on a Western blot. The presence of a ~120-kDa and a ~78-kDa band on Western blots with QGP+ and Bon1C membrane preparations implies that NIS protein is glycosylated by both QGP and Bon1 cells. The additional ~15-kDa band seen in QGP cells has previously been observed by other groups and suggests the presence of posttranscriptional modification or degradation of NIS protein (10, 22). As these processes are observed in neuroendocrine tumor cells that lack constitutive NIS protein expression, their physiological relevance remains to be determined.

Neuroendocrine tumor cells induced to express NIS accumulated iodide effectively, whereas control cells without NIS expression did not accumulate radioiodide. For radioiodide therapy, a high level of uptake in the tumor cells is a key factor because it contributes to the radiation dose to tumor tissue. We observed the highest levels of iodide uptake in QGP+ cells in which NIS expression is driven by the strong viral CMV promoter. Remarkably, the tissue specific chromogranin A promoter also induced NIS to a degree high enough to accumulate iodide 18-fold over control in Bon1C cells. In the thyroid, NIS concentrates iodide 20-fold over serum concentrations, which is sufficient for radioiodide therapy. With regard to the therapeutic potential of NIS gene transfer and to therapeutic gene transfer in general, there are two ways of obtaining expression of a therapeutic gene in target tissue while keeping expression low in other tissues. Either a vector can be used which is, by way of administration or because of other characteristics, specifically directed to the tumor (*e.g.*, intratumoral injection of adenoviruses or liposomes coated with tumor directed antigens). Alternatively, a nonspecific vector can be used with a promoter that restricts expression of the therapeutic gene to the tumor itself. Although the first approach allows the use of strong, nonspecific viral promoters such as the CMV promoter, two disadvantages limit its usefulness. First, in a situation with possibly widespread metastases, it will not be possible to reach and treat all tumor manifestations. Second, other tissues might express and be harmed by the therapeutic gene if administration is less than perfect. Both of these disadvantages do not apply to the second approach. By using a tumor-specific promoter such as the chromogranin A promoter with a nonspecific vector, small metastases and theoretically even single metastatic cells can be reached and eliminated. At the same time, although other tissues will receive the therapeutic gene, they will not express it and thus not be harmed. However, tissue/tumor-specific promoters tend to be weaker than viral promoters, and strength of expression of the therapeutic gene remains a concern. Most other groups using NIS in gene transfer have used viral promoters, demonstrating up to 225-fold accumulation of radioiodide in transfected cells. To date, there has been only one report of the successful use of a tissue specific promoter, the prostate-specific antigen promoter, in human prostatic adenocarcinoma cells (10). The authors reported a ~50-fold increase in iodide uptake and presented highly promising data on the therapeutic potential of this approach. We therefore present the second successful approach to tissue-specific NIS gene expression.

For effective radioisotope treatment, it is important to achieve a high radiation dose in the tumor while keeping the dose in the rest of the body low. The dose is determined by the level of uptake, *i.e.*, the level of NIS expression and the available amount of iodide, and the effective half-life of the isotope in the tumor, which is a product of the physical half-life (8.021 days for ^{131}I) and the biological half-life. The biological half-life depends on several factors: the rates of internalization and externalization, and whether the isotope is organified in the cell. We found rapid internalization of ^{131}I into Bon1C and QGP+ cells after NIS gene transfer, with maximal levels of uptake reached within 15 and 30 min, respectively. Fast internalization is desirable with view to a possible therapeutic use because it will facilitate effective concentration of radioactive iodide by the tumor even when ^{131}I is available in the serum for a limited amount of time. Efflux from QGP+ cells was rapid, with an effective $t_{1/2}$ of 5 min. Most other groups have reported half-lives of <10 min. Interestingly, iodide was externalized more slowly by Bon1C cells (effective $t_{1/2}$ 30 min). One other group has reported a similar retention time in monolayer breast cancer cells (16). As iodide is organified by the lactating mammary gland, we investigated whether organification of iodide could account for the long retention of iodide in Bon1C cells. However, no evidence was detected by HPLC and gel filtration chromatography. As the mechanism of iodide externalization from nonthyroid tissues is unknown, it remains unclear what accounts for the slow externalization of radioactive iodide from Bon1C cells.

^{131}I was highly effective in preventing the formation of QGP+ and Bon1C cell clones in the *in vitro* clonogenic assay. Clonogenic survival was reduced by three (99.9%) and two orders of magnitude (99%), respectively, after incubation with 100 μCi/ml ^{131}I. To our knowledge, this is the highest rate of reduction reported thus far. In contrast, clone formation was normal in control cell lines without NIS expression after incubation with ^{131}I as compared with mock-treated cells. This observation strongly supports that the reduction in clone formation is caused by internalized radioactive iodide, whereas radiation from iodide in the medium surrounding the cell appears to confer only minor, if any, damage. Even at lower activity concentrations (50 μCI/ml), we still observed impressive reductions in clone formation of 96 and 87% for QGP+ and Bon1C, respectively.

It is of interest to determine the absorbed dose for cells internalizing radioactive nuclides after NIS gene transfer in neuroendocrine tumor cells. Existing algorithms, however, are based on the use in three-dimensional systems such as tumor spheres or isolated, round cells. Any dose determination in monolayer cell culture where cells are spread out and thin can only be a rough estimate using these algorithms. It will be a valuable task to develop an algorithm suitable for the determination of absorbed dose in *in vitro* monolayer cell culture. Keeping the limitations of the approach we used in mind, it is still possible to arrive at some conclusions: (*a*) It is possible to deliver very high absorbed doses of up to 150 Gy to neuroendocrine tumor cells by NIS-mediated internalization of radioiodide. (*b*) Absorbed dose was considerably higher in QGP+ cells than in Bon1C, consistent with higher uptake and faster internalization. (*c*) D_0 was higher in QGP than in Bon1 cells, reflecting lower radiosensitivity and leveling out most of the effect of the higher absorbed dose in these cells.

We were able to demonstrate a cytotoxic effect of ^{131}I on Bon1C cells by PI staining. This is the first time that cytotoxicity has directly been shown after NIS gene transfer, and we consider it an important point regarding its possible therapeutic potential. Reduction in clonogenicity is not necessarily the result of cytotoxicity but may also be

achieved by inhibition of cell proliferation. However, in a therapeutic situation, cytotoxicity is mandatory to eliminate existing tumor cells. Most of the currently available therapeutic approaches for neuroendocrine tumors have not been shown to be cytotoxic. It is therefore important to demonstrate that radioiodide treatment after NIS gene transfer is indeed cytotoxic to pancreatic carcinoid cells.

Although our *in vitro* results are highly encouraging, it will be of critical importance whether they can be transferred to the *in vivo* situation. One aspect is the intrinsic radiosensitivity of the tumor. Generally, pancreatic neuroendocrine tumors are relatively indolent to external beam radiation, although there are reports of complete remission after radiotherapy (23). In contrast, other tumors such as differentiated thyroid cancer are sensitive to radiation in the form of radioiodide and external beam radiation in advanced disease (24). Theoretical considerations as well as observations made by other groups indicate that ^{131}I will be considerably more efficient in tissue than in monolayer cell culture. First, monolayers of cells measure ~5 μM in depth (25), whereas the mean range of ^{131}I β-particles is 700 μm. Only a tiny fraction of particles remains in the monolayer, whereas the majority leave the monolayer quickly and deposit their energy outside of the cells. Most tumors, in contrast, have diameters much larger than 700 μm, allowing for the complete absorption of β energy in the tumor. In addition, this crossfire effect helps to reach untransfected cells *in vivo*. Although all cells express NIS and accumulate ^{131}I in our experimental design, it is impossible to target and transfect 100% of tumor cells *in vivo*. However, untransfected cells can easily be destroyed by crossfire radiation from neighboring cells. Recently, these theoretical considerations have been elegantly supported by a study by Carlin *et al.* (26), who grew NIS-transfected UVW human glioma cells as 300-μm spheroids and monolayers and found a reduction in clonogenic survival of one log in spheroids as compared with monolayer cells after incubation with ^{131}I at various activity concentrations.

Finally, the effective half-life of radioiodide should be longer in *in vivo* tumors than in monolayer cells as a result of a higher iodide content in the microenvironment of tumor cells. The microenvironment of monolayer cells consists of medium in which the iodide concentration is low and equilibrium formation is unimpaired. In contrast, NIS-expressing tumor cells are surrounded by other cells, which concentrate and externalize iodide in turn. Therefore, the interstitial iodide concentration should be higher within the tumor, allowing cells to internalize iodide that has been released by others and slowing down equilibrium formation. This theory is supported by data from Spitzweg *et al.* (11), who reported effective half lives between 30 and 61.5 h for ^{131}I in xenografts of NIS-transfected prostate adenocarcinoma cells without evidence of organification. The long effective half-life most likely accounts for the highly encouraging *in vivo* data. In nude mice xenografts of NIS-expressing prostatic adenocarcinoma cells, complete remission was observed in 60% of animals after administration of 500 μCi of ^{131}I. The *in vitro* data from this study (98.75% reduction of clone formation) are of similar magnitude as those reported by Spitzweg *et al.* (75%), and it will be exciting to see whether *in vivo* results will be as promising in neuroendocrine tumor xenografts.

In conclusion, we present the first data on successful treatment of pancreatic neuroendocrine tumor cells with radioiodide after NIS gene transfer. Levels of NIS expression and iodide uptake are high both with the tissue specific chromogranin A and the CMV promoter. Using either approach, a striking reduction in clonogenicity as well as a direct cytotoxic effect on cells is observed. The approach clearly holds significant potential for the treatment of neuroendocrine tumors, provided that it can be transferred to the *in vivo* situation.

REFERENCES

1. Oberg, K. Neuroendocrine gastrointestinal tumors: a condensed overview of diagnosis and treatment. Ann. Oncol., *10:* S3–S8, 1999.
2. Arnold, R., Simon, B., and Wied, M. Treatment of neuroendocrine GEP tumours with somatostatin analogues: a review. Digestion, *62:* 84–91, 2000.
3. Frank, M., Klose, K. J., Wied, M., Ishaque, N., Schade-Brittinger, C., and Arnold, R. Combination therapy with octreotide and α-interferon: effect on tumor growth in metastatic endocrine gastroenteropancreatic tumors. Am. J. Gastroenterol., *94:* 1381–1387, 1999.
4. Arnold, R., Wied, M., and Behr, T. Somatostatin analogues in the treatment of endocrine tumours of the gastrointestinal tract. Exp. Opin. Pharmacother., *3:* 643–656, 2002.
5. Oberg, K. Chemotherapy and biotherapy in the treatment of neuroendocrine tumours. Ann. Oncol., *12:* S111–S114, 2001.
6. Chow, S. M., Law, S. C., Mendenhall, W. M., Au, S. K., Chan, P. T., Leung, T. W., Tong, C. C., Wong, I. S., and Lau, W. H. Papillary thyroid carcinoma: prognostic factors and the role of radioiodine and external radiotherapy. Int. J. Radiat. Oncol. Biol. Phys., *52:* 784–795, 2002.
7. Cho, J. Y., Xing, S., Liu, X., Buckwalter, T. L., Hwa, L., Sferra, T. J., Chiu, I. M., and Jhiang, S. M. Expression and activity of human Na+/I- symporter in human glioma cells by adenovirus-mediated gene delivery. Gene Ther., *7:* 740–749, 2000.
8. Boland, A., Ricard, M., Opolon, P., Bidart, J. M., Yeh, P., Filetti, S., Schlumberger, M., and Perricaudet, M. Adenovirus-mediated transfer of the thyroid sodium/iodide symporter gene into tumors for a targeted radiotherapy. Cancer Res., *60:* 3484–3492, 2000.
9. Mandell, R. B., Mandell, L. Z., and Link, C. J., Jr. Radioisotope concentrator gene therapy using the sodium/iodide symporter gene. Cancer Res., *59:* 661–668, 1999.
10. Spitzweg, C., Zhang, S., Bergert, E. R., Castro, M. R., McIver, B., Heufelder, A. E., Tindall, D. J., Young, C. Y., and Morris, J. C. Prostate-specific antigen (PSA) promoter-driven androgen-inducible expression of sodium iodide symporter in prostate cancer cell lines. Cancer Res., *59:* 2136–2141, 1999.
11. Spitzweg, C., Dietz, A. B., O'Connor, M. K., Bergert, E. R., Tindall, D. J., Young, C. Y., and Morris, J. C. *In vivo* sodium iodide symporter gene therapy of prostate cancer. Gene Ther., *8:* 1524–1531, 2001.
12. Goddu, S. M., Howell, R. W., Bouchet, L. G., Bolch, W. E., and Rao, D. V. MIRD cellular S values: self-absorbed dose per unit cumulated activity for selected radionuclides and monoenergetic electron and α particle emitters incorporated into different cell compartments, p. 183. Society of Nuclear Medicine, 1997.
13. Spitzweg, C., Heufelder, A. E., and Morris, J. C. Thyroid iodine transport. Thyroid, *10:* 321–330, 2000.
14. Heufelder, A. E., Morgenthaler, N., Schipper, M. L., and Joba, W. Sodium iodide symporter-based strategies for diagnosis and treatment of thyroidal and nonthyroidal malignancies. Thyroid, *11:* 839–847, 2001.
15. Haberkorn, U., Henze, M., Altmann, A., Jiang, S., Morr, I., Mahmut, M., Peschke, P., Kubler, W., Debus, J., and Eisenhut, M. Transfer of the human NaI symporter gene enhances iodide uptake in hepatoma cells. J. Nucl. Med., *42:* 317–325, 2001.
16. Nakamoto, Y., Saga, T., Misaki, T., Kobayashi, H., Sato, N., Ishimori, T., Kosugi, S., Sakahara, H., and Konishi, J. Establishment and characterization of a breast cancer cell line expressing Na+/I- symporters for radioiodide concentrator gene therapy. J. Nucl. Med., *41:* 1898–1904, 2000.
17. Shimura, H., Haraguchi, K., Miyazaki, A., Endo, T., and Onaya, T. Iodide uptake and experimental 131I therapy in transplanted undifferentiated thyroid cancer cells expressing the Na+/I- symporter gene, Endocrinology, *138:* 4493–4496, 1997.
18. Smit, J. W., Schroder-van der Elst, J. P., Karperien, M., Que, I., Romijn, J. A., and van der Heide, D. Expression of the human sodium/iodide symporter (hNIS) in xenotransplanted human thyroid carcinoma. Exp. Clin. Endocrinol. Diabetes, *109:* 52–55, 2001.
19. Spitzweg, C., Joba, W., Eisenmenger, W., and Heufelder, A. E. Analysis of human sodium iodide symporter gene expression in extrathyroidal tissues and cloning of its complementary deoxyribonucleic acids from salivary gland, mammary gland, and gastric mucosa. J. Clin. Endocrinol. Metab., *83:* 1746–1751, 1998.
20. Spitzweg, C., Joba, W., and Heufelder, A. E. Expression of thyroid-related genes in human thymus. Thyroid, *9:* 133–141, 1999.
21. Vayre, L., Sabourin, J. C., Caillou, B., Ducreux, M., Schlumberger, M., and Bidart, J. M. Immunohistochemical analysis of Na+/I- symporter distribution in human extra-thyroidal tissues. Eur. J. Endocrinol., *141:* 382–386, 1999.
22. Castro, M. R., Bergert, E. R., Beito, T. G., McIver, B., Goellner, J. R., and Morris, J. C. Development of monoclonal antibodies against the human sodium iodide symporter: immunohistochemical characterization of this protein in thyroid cells. J. Clin. Endocrinol. Metab., *84:* 2957–2962, 1999.
23. Tennvall, J., Ljungberg, O., Ahren, B., Gustavsson, A., and Nillson, L. O. Radiotherapy for unresectable endocrine pancreatic carcinomas. Eur. J. Surg. Oncol., *18:* 73–76, 1992.
24. Brierley, J. D., and Tsang, R. W. External-beam radiation therapy in the treatment of differentiated thyroid cancer. Semin. Surg. Oncol., *16:* 42–49, 1999.
25. Bettega, D., Calzolari, P., Doglia, S. M., Dulio, B., Tallone, L., and Villa, A. M. Technical report: cell thickness measurements by confocal fluorescence microscopy on C3H10T1/2 and V79 cells. Int. J. Radiat. Biol., *74:* 397–403, 1998.
26. Carlin, S., Cunningham, S. H., Boyd, M., McCluskey, A. G., and Mairs, R. J. Experimental targeted radioiodide therapy following transfection of the sodium iodide symporter gene: effect on clonogenicity in both two-and three-dimensional models. Cancer Gene Ther., *7:* 1529–1536, 2000.

Radioiodide Treatment after Sodium Iodide Symporter Gene Transfer Is a Highly Effective Therapy in Neuroendocrine Tumor Cells

Meike L. Schipper, Alexander Weber, Martin Béhé, et al.

Cancer Res 2003;63:1333-1338.