Visualization of Endogenous p53-Mediated Transcription In vivo Using Sodium Iodide Symporter

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ABSTRACT

Purpose: To develop a gamma camera imaging method for the determination of endogenous gene expression, we evaluated the expression of endogenous p53 gene using human sodium iodide symporter (hNIS) gene as reporter.

Experimental Design: We constructed cis-p53RE-hNIS reporter vector placed under control of an artificial enhancer (p53RE). Moreover, we transfected it into human hepatoma cell line SK-Hepl by liposome. Geneticin was used for the selection of stable transfectant (SK-Heplp53NIS). To evaluate the function of hNIS, the inhibition study was examined with 1 mmol/L potassium perchlorate. After treatment of Adriamycin with serial dose for 24 hours, we measured the uptake of 125I and did Western blot analysis to evaluate expression of p53 protein. Tumor xenografts were produced in nude mice by s.c. injection of SK-Heplp53NIS cells. After 7 days, scintigraphic images of nude mice before and after Adriamycin treatment were obtained using [99mTc]-pertechnetate.

Results: In the SK-Heplp53NIS cells, Adriamycin-treated cells accumulated up to three times higher than did nontreated cells. Potassium perchlorate inhibited completely the uptake of 125I. As Adriamycin dose increased, radiiodide uptake was significantly correlated with activated p53 as well as total p53 protein level. When Adriamycin (2 mg/kg) was treated in the same mice, a significantly higher level can be monitored by using a simple gamma camera; no immune response is expected because NIS is an endogenously expressed gene; and NIS has some advantages versus other positron emission tomography nuclear imaging reporter genes such as HSV1-tk or D2R.

Conclusions: These results suggest that p53 expression level can be monitored by NIS gene expression using cis-p53RE-hNIS system in vitro and in vivo.

INTRODUCTION

Sodium iodide symporter (NIS) is expressed mainly in the thyroid, salivary gland, and gastric mucosa and can transport a variety of anions such as iodide, pertechnetate, and perrhenate (1–3). NIS is an intrinsic membrane glycoprotein with 13 putative transmembrane domains and allows the thyroid gland to transport and concentrate iodide at 20- to 40-fold above the plasma concentration (4).

Imagings and uptake tests with radioiodide and [99mTc]-pertechnetate have been used clinically to evaluate the thyroid diseases. The cloning of the hNIS gene gives us a possibility of using hNIS therapeutically to concentrate 131I or 188Re by gene transfer into cells or animal tumor models (5–10). hNIS may also serve as an ideal imaging reporter for the visualization of endogenous and exogenous gene expression (11, 12).

NIS has some advantages versus other positron emission tomography nuclear imaging reporter genes such as HSV1-tk or D2R: (a) NIS readily accumulates commonly used radioisotopes like 131I, 125I, 123I, and 99mTc and does not require complicated radiocompounds; (b) scintigraphy of NIS can be acquired by using a simple gamma camera; (c) no immune response is expected because NIS is an endogenously expressed gene; and (d) NIS has been used clinically for >50 years and its physiologic effects are well known (12).

A noninvasive method for imaging the activities of different signal transduction pathways and the expressions of different genes in vivo would be of considerable value. It would aid our understanding of the roles of specific genes and signal transduction pathways in various diseases and could be used to elucidate the dynamics and temporal regulation of gene expression at different disease stages during therapeutic intervention. Several imaging studies, including p53-dependent gene expression and the T-cell receptor–dependent activation of T lymphocytes by positron emission tomography, have shown the usefulness of noninvasive molecular imaging methods for assessing therapeutic approaches (13, 14).

We undertook to develop a gamma camera imaging method to show endogenous p53 expression using hNIS gene. The p53 tumor suppressor gene was selected as a model gene for these imaging studies because it is commonly mutated in human cancer (15, 16). The SK-Hepl cell line, which expresses wild-type p53, was selected for this study. The SK-Hepl cell line was transfected with the cis-p53RE-hNIS reporter system in which the hNIS reporter gene is under the control of an artificially constructed cis-acting p53-specific enhancer. We found that the induction of p53 can be monitored by 125I uptake assay in vitro and by the gamma camera imaging of xenografts in vivo.
MATERIALS AND METHODS

Construction of p53RE-hNIS Vectors. hNIS cDNA was inserted into the plasmid pIRE2-EGFP (Clontech, Palo Alto, CA). The hNIS gene was released by XhoI and MluI restriction enzymes from the plasmid (kindly provided by Dr. Sissy M. Jhiang, Ohio State University, Columbus, OH) and ligated to pIRE2-EGFP vector backbone (pIRE2-hNIS). The cytomegalovirus promoter located between AseI and NheI sites was removed from the pIRE2-hNIS plasmid, and a p53 enhancer element containing a TATA box from the plasmid p53-Luc (Stratagene, La Jolla, CA) was prepared by PCR using the following primers: forward primer 5’-ACGCACTTAAATGTCGGATCCGAAGCTCC-3’ and reverse primer 5’-CTAGCTAGCTATATACCCTCTAGAGTCTC-3’. PCR products were released by AseI and NheI and then inserted into the plasmid pIRE2-hNIS above (p53RE-hNIS).

Establishment of Stably Transfected SK-Hep1 Cell Lines. Human hepatoma cells SK-Hep1 (wild-type p53) were grown in RPMI 1640 containing 100,000 IU/L penicillin, 100 μg/mL streptomycin, 250 μg/mL amphotericin B, and 10% (v/v) fetal bovine serum (Invitrogen, Grand Island, NY). Cells were transfected with the p53RE-hNIS vector using LipofectAMINE Plus (Invitrogen, Carlsbad, CA). Selection was done using 300 to 800 μg/mL gentamicin (Invitrogen, Grand Island, NY) in RPMI 1640 containing 10% fetal bovine serum for 2 weeks, beginning the day after the transfection. Surviving clones were isolated and screened for p53-dependent iodide uptake activity. Adriamycin was used to induce the expression of the endogenous p53 gene. After inducig p53 with Adriamycin (0.5 μmol/L) for 24 hours, SK-Hep1p53NIS, the clone with the highest p53-dependent iodide uptake activity, was chosen for the following studies. The optimal concentration (0.5 μmol/L) of Adriamycin required for highest p53 expression was determined as described by Vyasasdee et al. (17). To confirm the NIS function, potassium perchlorate was used as an inhibitor.

In vitro 125I Uptake Assay. SK-Hep1 cells stably transfected with the reporter vector (SK-Hep1p53NIS) were plated at a cell density of 3 × 10^5 cells per well in a 24-well plate and cultured for 12 hours with RPMI 1640 containing 10% fetal bovine serum. The cells were then treated in quadruplicates with Adriamycin (0.0125, 0.025, 0.05, 0.1, or 0.5 μmol/L) to activate the p53 pathway, and 24 hours later, the clones were assayed for NIS activity by radioiodide uptake at 37°C using a modification of the method described by Weiss et al. (18). In brief, the iodide uptake level was determined by incubating the cells with 500 μL HBSS containing 0.5% bovine serum albumin and 10 mmol/L 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid-NaOH (pH 7.4), with a 3.7 kBq (0.1 μCi) carrier-free Na[125]I and 10 μmol/L NaI, to yield a specific activity of 740 MBq/mmol (20 mCi/mmol) at 37°C for 30 minutes. After incubation, the cells were washed twice as quickly as possible (>15 seconds) with 2 mL iodine-free, ice-cold HBSS buffer. The cells were detached with 500 μL trypsin, and radioactivity was measured using a gamma counter (Cobra II, Canberra Packard, Meriden, CT).

Reverse Transcription-PCR Analysis for hNIS. Total RNA was prepared from SK-Hep1p53NIS cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol with slight modification. Briefly, total RNA (~500 ng) was reverse transcribed in a final volume of 20 μL containing oligo(dT) (1 μL), 5× first strand buffer (4 μL), 0.1 mol/L DTT (2 μL), 10 mmol/L deoxynucleotide triphosphate mix (1 μL), and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Grand Island, NY). The following PCR was done in a total volume of 20 μL containing cDNA (2 μL), 10× reaction buffer (2 μL), forward primer (5’-TCTCTCACTAAGCCCTCT-3’) and reverse primer (5’-ATCCAGGATGGCCACTTCT-3’), 10 mmol/L deoxynucleotide triphosphate (1 μL), and Taq-DNA polymerase (2.5 units, GeneCraft, Münster, Germany) using a GeneAmp PCR system (Applied Biosystems, Foster City, CA). The samples were then subjected to 5 minutes of denaturation at 94°C, 25 amplification cycles (30 seconds at 94°C, 30 seconds at 50°C, and 1 minute at 72°C), and an additional 5 minutes at 72°C.

β-actin was amplified as a control using the same reaction solution but with β-actin primers forward (5’-TGAGGCGGTGCAACCCACACTGTTGCCATCA-3’) and reverse (5’-CTAAGAGCCATTTGGGGAGGATGAGGAC-3’). The samples were subjected to 5 minutes of denaturation at 94°C, 23 PCR cycles (30 seconds at 94°C, 30 seconds at 50°C, and 1 minute at 72°C). The amplified products were analyzed on ethidium bromide-stained agarose gel electrophoresis. Results were interpreted using TINA 2.1 (Raytest, Straubenhardt, Germany). Expression values of the NIS gene were calculated by dividing NIS band intensities by that of β-actin. The analysis of the correlation between gene expression (by reverse transcription-PCR) and protein activity (by radioiodide uptake) was carried out using Sigma Plot 2001 (SPSS, Inc., Chicago, IL).

Western Blot Analysis. Cells were harvested with a scraper after being washed with ice-cold PBS and lysed in a buffer containing 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L DTT, 20% (v/v) glycerol, 1 mmol/L EDTA, and protease inhibitor mixture. The samples were then centrifuged at 4°C, 10,000 × g for 10 minutes. The supernatant was mixed with SDS sample buffer containing 2× Laemmli loading buffer, 15% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue, and 0.2% SDS, heated to 95°C for 5 minutes, and the samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies against human p53 (Ab-6, 1:1,000 dilution, Oncogene, San Diego, CA), human β-actin (clone A5448, 1:2,000 dilution, Sigma, St. Louis, MO) rabbit anti-mouse secondary antibody, and horseradish peroxidase conjugated secondary antibody. Chemiluminescence reagents were detected using the ChemiDoc XRS (Bio-Rad, Hercules, CA). Protein concentrations in samples were assayed using a BCA Protein Assay kit (Pierce, Rockford, IL).

Generation of s.c. Tumor Xenografts in Nude Mice. All animal experiments were done with the approval of the Seoul National University Animal Research Committee. s.c. xenografts were produced in male BALB/c nu/nu mice by injecting (s.c.) different numbers of cells suspended in 100 μL RPMI
show that the increased $^{125}$I uptake was due to a competitive inhibitor of NIS (Fig. 1). These results clearly was completely inhibited by $30$ mol/L potassium perchlorate. Thirty minutes after injecting $18.5$ MBq (0.5 mCi) $[^{99m}$Tc]-pertechnetate per animal into a lateral tail vein, mice were placed in a spread prone position and scanned with a gamma camera (ON 410, Ohio Nuclear, Solon, OH) equipped with a pinhole collimator. The next day, the animals received a $0.2$ mL i.p. injection of $2$ mg/kg Adriamycin to induce the p53 pathway in the xenografts. Scintigraphic images of the nude mice were obtained using $[^{99m}$Tc]-pertechnetate, as described above, $24$ hours after Adriamycin administration.

RESULTS

Generation of cis-p53RE-hNIS Expressing SK-Hep1 Cell Lines. After transfecting SK-Hep1 cells with cis-p53RE-hNIS reporter vector using liposome and their selection with geneticin, cell lines stably expressing hNIS controlled by p53-specific enhancer were established. To investigate hNIS activity in the transfected SK-Hep1 cell lines, iodide uptake experiments were done. After treating cells with $0.5$ μmol/L Adriamycin for $24$ hours, $^{125}$I uptake in the transfected SK-Hep1 cell lines (six cell lines) was found to be 9- to 26-fold higher than that of the wild-type SK-Hep1. The cell line SK-Hep1p53NIS showing highest $^{125}$I uptake was selected for further experiments. In addition, $^{125}$I uptake was completely inhibited by $30$ μmol/L potassium perchlorate, a competitive inhibitor of NIS (Fig. 1). These results clearly show that the increased $^{125}$I uptake was due to hNIS gene expression.

Characterization of the Iodide Uptake Capacity of cis-p53RE-hNIS Transfected Cells In vitro. Some background p53 activity, represented by $^{125}$I uptake, was observed in the nontreated SK-Hep1p53NIS cells, which was probably cell cycle associated. Radioiodide uptake was found dependent on the Adriamycin concentration, and $0.5$ μmol/L Adriamycin showed maximal activation of the p53 signaling pathway and highest hNIS levels (Fig. 1).

Up-Regulation of p53 in Adriamycin-Treated SK-Hep1p53NIS Cells. Significant increases in phosphorylated p53 and total p53 protein expression during Adriamycin treatment were observed by Western blotting with p53-specific antibody (Fig. 2). Increased p53 activity caused the up-regulation of hNIS gene expression as manifested by hNIS mRNA expression (Fig. 3). Increased hNIS mRNA in Adriamycin-treated SK-Hep1p53NIS cells corresponded with significantly higher levels of $^{125}$I uptake versus nontreated SK-Hep1p53NIS cells. In contrast, SK-Hep1 cells showed no increased $^{125}$I uptake after Adriamycin treatment. Good correlations were observed between activated p53 ($r^2 = 0.795$) and total p53 expression ($r^2 = 0.908$) and radioiodide uptake in this in vitro model (Fig. 4).

DISCUSSION

Whereas conventional positron emission tomography imaging reporter genes, such as HSV1-Ik and D2R, require the synthesis of complicated substrates and the availability of expensive positron emission tomography equipment, NIS has widely available substrates, radioiodines and $[^{99m}$Tc]-pertechnetate, which can be visualized by a gamma camera. Moreover, the clearance of these substrates from the body is well understood, and radioiodines or $[^{99m}$Tc]-pertechnetate present no labeling instability problems, which may be a major concern for radiolabeled ligands of D2R. In addition, the NIS gene is unlikely to interact with the underlying cell biochemistry, iodide is not metabolized in most tissues, and although sodium influx
may be a concern, no adverse effects have been observed to date (12, 19).

The direct imaging of each gene or step of different signal transduction pathways present practical limitations with respect to the development of many unique probes. These limitations

![Fig. 2](image1) Western blot analysis for total p53 and activated p53 (p53 Ser15) protein levels and their dependence on Adriamycin dose in SK-Hep1p53NIS cells. SK-Hep1p53NIS cells were treated with 0-0.5 μmol/L Adriamycin to induce p53 expression. After 24 hours of Adriamycin treatment, total protein was extracted. Total p53 and activated p53 (p53 Ser15) proteins were immunoblotted with anti-p53 and anti- phosphorilated p53. Expressions of total p53 and activated p53 protein were increased in a dose-dependent manner by Adriamycin treatment in SK-Hep1p53NIS cells.

![Fig. 3](image2) Dependence of hNIS expression on Adriamycin dose in SK-Hep1p53NIS cells. Quantitative reverse transcription-PCR analysis for hNIS mRNA was done in the samples described in Fig. 2. At 24 hours after Adriamycin treatment, RNA was extracted and total RNA (0.5 μg) was reverse transcribed and amplified by using hNIS-specific primer. hNIS mRNA levels were normalized with β-actin. Increased p53 activity caused the up-regulation of hNIS gene expression as manifested by hNIS mRNA expression.

![Fig. 4](image3) Correlation between 125I uptake and p53 protein levels. Correlation between 125I uptake and p53 expression level was investigated by Western blot at different Adriamycin doses. There were good correlations between activated p53 ($r^2 = 0.795$) and total p53 ($r^2 = 0.908$) expression and radioiodide uptake in this in vitro model. Increased p53 activity by Adriamycin caused the up-regulation of hNIS gene expression and increased 125I uptake.
same experiments with the same reporter vector in human anaplastic thyroid cancer cells expressing mutant p53, ARO (data not shown). As our expectation, there is no induction by Adriamycin treatment. These results suggest that Adriamycin does not have any effect directly on NIS expression.

We found that the levels of hNIS reporter gene mRNA paralleled those of p53 protein and phosphorylated p53 protein in a dose-dependent manner in both noninduced and induced states. Similarly, very close relation was observed between radioactive uptake and hNIS mRNA levels. In addition, the up-regulation of hNIS expression in SK-Hep1p53NIS xenografts was observed after Adriamycin treatment using a gamma camera in vivo. These results show that the cis-p53RE-hNIS reporter system adequately reflects the activity of the p53 signal transduction pathway in vitro and in vivo.

In this study, we used [99mTc]-pertechnetate instead of radioiodines as a NIS substrate for in vivo imaging. It has been reported that NIS transports several anions like $\text{ClO}_4^-$, $\text{TcO}_4^-$, $\text{ReO}_4^-$, SCN$^-$, $\text{ClO}_3^-$, and Br$^-$ as well as iodide (I$^-$); monovalency and anion size are the determinants of NIS substrates (37). We obtained scintigraphic images using [99mTc]-pertechnetate, which has a short half-life (6 hours), before and after Adriamycin treatment in the same mice. Our results suggest that gamma camera imaging methods in combination with a hNIS reporter system may be helpful for the daily monitoring of reporter activity both in the target tissue and in the body (11).

Although this study shows the feasibility of using hNIS as a reporter gene for monitoring the transcriptional activation of endogenous genes by gamma camera imaging, the hNIS reporter system has several disadvantages: (a) background signals are high in the thyroid and stomach; thus, it is recommended that another imaging reporter system be used if the target organ is near organs showing high background.

**Fig. 5** Scintigraphic image of endogenous p53 activation. Tumors were established in vivo by s.c. injection into four different nude mice sites: $1 \times 10^7$ of SK-Hep1, left shoulder (negative control); SK-Hep1p53NIS, right shoulder ($5 \times 10^6$); SK-Hep1p53NIS, left thigh ($1 \times 10^7$); and SK-Hep1p53NIS, right thigh ($2 \times 10^7$). After 7 days, planar scintigraphic images of [99mTc]-pertechnetate uptake before and after treatment of Adriamycin (2 mg/kg) in the same mouse were obtained. Thirty minutes after the injection of 0.5 mCi [99mTc]-pertechnetate per animal, the mice were scanned with a gamma camera equipped with a pinhole collimator. Scintigraphies showed increased uptake of [99mTc]-pertechnetate in test tumors (SK-Hep1p53NIS) after Adriamycin treatment compared with the control tumor (SK-Hep1).

**Fig. 6** Intensity ratio of SK-Hep1p53NIS to SK-Hep1 in mice ($n = 5$) before and after Adriamycin treatment. The intensity of [99mTc]-pertechnetate uptake was analyzed with Tina 2.1 using planar scintigraphic images of Fig. 5. When Adriamycin was given to these mice, significantly higher [99mTc]-pertechnetate uptake was observed in SK-Hep1p53NIS than in SK-Hep1 xenografts ($P < 0.05$, paired $t$ test) or in nontreated SK-Hep1p53NIS xenografts ($*, P < 0.05$, unpaired $t$ test).
activity. (b) Radioiodide accumulated by NIS can flow out rapidly, which minimizes its harmful effect on target tissue. The determination of acquisition time is needed in terms of the uptake measurement and NIS efflux kinetics. (c) Reporter gene activity in vivo has a weaker signal than enzymatic reporter genes, which trap reporter probes within cells. Thus, adequate amplification strategies are necessary, such as a two-step transcription amplification system (38), to improve in vivo imaging at the molecular level.

In summary, the present study shows that gamma camera–based imaging of the [125I]pertechnetate/cis-p53RE-hNIS reporter system is sufficiently sensitive to detect the transcriptional up-regulation of genes in the p53 signal transduction pathway. This gamma camera/cis-p53RE-hNIS reporter system imaging of the transcriptional activity of p53 in tumors may be used to assess new anticancer drugs or novel therapeutic approaches that depend on p53 activation.

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