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Adenovirus-mediated and Targeted Expression of the Sodium Iodide Symporter (NIS) permits *In Vivo* Radioiodide Imaging and Therapy of Pancreatic Tumors

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The abbreviations used are: NIS, sodium iodide symporter; CMV, cytomegalovirus; IV, intravenous; MOI, multiplicity of infection.

Abstract

Pancreatic cancer is the fourth leading cause of cancer death in the United States. It is highly aggressive with no uniformly effective chemotherapy available for metastatic disease. The Sodium Iodide Symporter (NIS) is a transmembrane protein responsible for uptake of iodide into cells. The presence of NIS in thyroid cells permits diagnostic imaging and therapy of thyroid tumors using radioiodide. Previous studies from this laboratory reported MUC1-driven expression of NIS in cancer cells. MUC1 overexpression has also been reported in 90% of pancreatic tumors. In this study Ad5/MUC1/NIS was used to infect pancreatic cancer cells both *in vitro* and *in vivo*, to investigate the potential for radioiodide imaging and ablation of this disease. *In vitro* studies revealed a 43-fold increase in iodide uptake in NIS-transduced cells compared to controls. *In vivo* imaging revealed effective iodide uptake and retention at the site of NIS-transduced tumors, with optimal uptake (13% of injected dose) observed 5 hrs after iodide administration. IV delivery was performed to investigate potential hepatotoxicity of the construct in the event of virus leakage. IV injection of Ad5/CMV/NIS resulted in robust iodide uptake throughout the mouse liver, while no uptake was detected in the liver of animals given IV Ad5/MUC1/NIS. Administration of therapeutic doses of ^{131}I resulted in significant regression of NIS-transduced tumors, with a mean 50% reduction in volume within 10 weeks of therapy ($P<0.0001$). The ability to target NIS expression to pancreatic cancer, which has such limited treatment options, may be highly beneficial and warrants further investigation.

Overview summary

The ability of thyroid cells to actively accumulate iodide via the sodium iodide symporter (NIS) forms the basis for the use of radioiodide to image and treat thyroid disease. Transfer of NIS expression to other tumor types would potentially provide for use of this effective and non-invasive therapy to treat diseases of non-thyroid origin. MUC1 is overexpressed in many tumor types including breast, pancreatic, lung and ovarian. Transcriptional targeting of NIS expression using the MUC1 promoter limits expression of the gene to MUC1-positive tumor cells, a cohort which includes >90% of pancreatic cancers. This study is an important initial step investigating the potential for use of radioiodide as a systemic therapeutic agent in pancreatic cancer treatment. The results presented clearly demonstrate that the application of this virus is not limited to one tumor type, but may provide for imaging and therapy of a range of MUC1-positive malignancies.

Introduction

Pancreatic cancer is a highly aggressive disease with an increasing incidence worldwide. Early symptoms of the disease are vague, so it is usually diagnosed at an advanced stage with a high incidence of associated metastases, and has an average 5-year survival of <5% (MacKenzie, 2004). The urgent need for new therapeutic agents has fueled advances in the molecular understanding of the disease and led to the identification of many potential therapeutic targets (MacKenzie, 2004; Moniaux *et al.*, 2004; Xiong, 2004). It has become clear that the development and spread of pancreatic cancer is driven by multiple genetic and epigenetic changes and so restoration or inhibition of one gene or pathway may not be sufficient to conquer this complex malignant disease.

Overexpression of the MUC1 gene has been associated with the development, progression and metastasis of pancreatic cancer (Yonezawa and Sato, 1997; Moniaux *et al.*, 2004). MUC1 is a transmembrane glycoprotein expressed at the apical surface of secretory epithelial cells (Gendler, 2001). In the pancreas, MUC1 expression is confined to the apical membrane of intralobular ductules, and is only weakly expressed in the normal adult pancreas (Yonezawa and Sato, 1997; Moniaux *et al.*, 2004). However, in pancreatic cancer MUC1 expression is greatly increased and is no longer restricted to the apical surface, but is found throughout the cell, with an altered glycosylation pattern (Mukherjee *et al.*, 2000). Although it is not fully understood, this deregulated expression pattern is thought to disturb cell-cell/cell-matrix interactions, thus decreasing cell adherence and favoring release of malignant cells into the circulation. Hence, overexpression of MUC1 confers an apparent advantage to malignant cells and is a

marker of an aggressive cancer phenotype (Levi *et al.*, 2004), making it an attractive therapeutic target (Miles and Papadimitriou, 1999; Pecher *et al.*, 2002).

We recently described the use of the MUC1 promoter to drive expression of the sodium iodide symporter (NIS) in breast and ovarian cancer xenografts in nude mice (Dwyer *et al.*, 2005a,b). NIS is a transmembrane glycoprotein that permits active uptake of iodide from the bloodstream (Dohan *et al.*, 2003). Native NIS expression in the thyroid gland has permitted the use of radioiodide to image and treat thyroid disease for many years (Mazzaferri and Kloos, 2001). The presence of NIS in other tissues has raised the possibility of using this safe and effective treatment modality for the ablation of tumors of non-thyroid origin (Dohan and Carrasco, 2003). In the breast cancer model described previously, successful MUC1-driven NIS gene transfer resulted in effective imaging and treatment of breast tumor xenografts in an animal model, resulting in a >80% reduction in tumor volume (Dwyer *et al.*, 2005a). The fact that the MUC1 promoter is not specific to breast cancer means that this construct may have potential applications in other tumor types (Chen *et al.*, 1996; Teoh *et al.*, 1998; Block *et al.*, 2002; Mullen *et al.*, 2002; Gupta *et al.*, 2003; Dwyer *et al.*, 2005b). Since increased MUC1 expression has been reported in 90% of pancreatic cancers (Mukherjee *et al.*, 2000), and is associated with an aggressive phenotype for which there is currently no effective therapy, this MUC1/NIS construct may be well suited to this tumor type. The aim of this study was to introduce membrane-targeted functional NIS expression in pancreatic tumors and potentially provide for non-invasive imaging and radioiodide ablation of this aggressive malignancy.

Materials and Methods

Cell Culture. The MUC1-positive pancreatic cancer cell line, Capan-2 was maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum and penicillin G (2 units/ml)/ streptomycin sulfate (100 mg/ml). Cells were maintained at 37°C, 5% CO_2 with a medium change twice weekly and passage every seven days.

Adenovirus Production. A replication-deficient human recombinant adenovirus serotype 5 construct containing human NIS under the control of the MUC1 promoter (-1401 to +33) was produced in collaboration with ViraQuest Inc. (North Liberty, Iowa) as described previously (Dwyer *et al.*, 2005a). A replication deficient adenovirus containing NIS under the control of the non-specific CMV promoter, and a promoter-free virus (control) were in stock from previous experiments (Dwyer *et al.*, 2005a) 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 2.5 x 10^5 cells/well, to reach ~60% confluence for infection. Cells were then washed and incubated in serum-free medium and the appropriate MOI of virus added to each well. Following a 24 hr incubation at 37°C, cells were washed and incubated in complete medium for 72, 96 or 120 hrs before analysis of NIS expression/function. All adenoviral infections were carried out at least in triplicate.

^{125}I Uptake Studies. The ability of NIS transduced Capan-2 cells to concentrate iodide was determined as described by Weiss et al. (1984). Briefly, following transfection, cells

were incubated in HBSS containing 10 mM HEPES, 10 μM NaI and 0.1 μCi of $Na^{125}I$/ml, pH 7.3, and 10 μM $KCLO_4$, a competitive inhibitor of iodide uptake by NIS, was included in control wells. Following a 45-min incubation at 37°C, cells were washed with ice-cold PBS, lysed with 1 M NaOH, and concentrated iodide was measured on a γ-counter.

^{125}I Efflux Studies. The rate of iodide efflux from cells was determined as described by Weiss et al. (1984). Cells were trypsinized into 6-well plates and incubated with ^{125}I as described for the iodide uptake assay. After rinsing twice in ice cold PBS, 1 ml of warm PBS was added to each well. After 5 mins this was removed to a pre-labeled tube and replaced with another 1ml of warm PBS. This procedure was repeated until an aliquot of PBS had been added/removed every 5 mins for the required time period after which the remaining ^{125}I was removed by lysing the cells in 1 M NaOH for 10 mins at room temperature. The amount of iodide in each time point sample was then counted on a γ-counter.

Growth of Pancreatic Cancer Xenografts in Mice. Male athymic nude mice (Harlan Sprague-Dawley, Indianapolis, IN, USA) received a subcutaneous injection of 1 x 10^7 Capan-2 cells suspended in 0.2 ml McCoy's 5A medium in one or both flanks. For imaging experiments, animals were implanted with tumors on both flanks and injected with a different virus in each for comparison. In the case of ^{131}I therapy, mice were injected with only one tumor, as growth of control tumors would potentially result in disease progression/spread and affect survival of animals injected with Ad/CMV/NIS or

Ad/MUC1/NIS. When tumors had reached an appropriate volume (≥100 mm^3), mice were given T_4 supplemented water (5 mg/L) to reduce thyroidal uptake of iodide, and placed on a low iodine diet to reduce competitive inhibition of radioiodide uptake at the tumor site. All experiments were approved and carried out according to the guidelines of the Animal Care and Use Committee of the Mayo Clinic, Rochester, MN.

***In Vivo* Adenoviral Infections.** Mice with tumors ≥100 mm^3 that had been maintained on the low iodide diet were given intra-tumoral injections of recombinant Ad/MUC1/NIS or Ad/CMV/NIS or control virus in a total volume of 100 μl 3% sucrose/phosphate buffered saline. In an initial experiment injection doses were varied from 5 x 10^8 – 2 x 10^9 pfu, and were then kept constant at the optimum dose of 1 x 10^9 pfu in all remaining experiments. Tumors were injected at multiple sites to increase virus dispersal, using insulin syringes with 28-gauge needles. In order to investigate potential hepatotoxicity of the construct in the event of virus leakage, mice were also given an intravenous injection of 1 x 10^9 pfu of either the non-specific Ad/CMV/NIS or the tumor targeted Ad/MUC1/NIS.

***In Vivo* Tumor Imaging.** Four days following virus injection, mice (n=18) were given an intraperitoneal injection of 0.5 mCi ^{123}I and images were acquired on a gamma camera with a low energy, high resolution collimator (Helix System; Elscint, Haifa, Israel). A four day interval was chosen based on *in vitro* results showing optimal NIS expression in this cell line four days following virus infection (Fig. 1). Serial images of all animals, in

prone position, (n=18) were acquired at 1, 3, 5, 7, 24 and 48 hrs following iodide administration to investigate the rate of iodide efflux from the tumors.

^{131}I Therapy. Mice were grouped according to the size of the Capan-2 tumors and given intra-tumoral injections of virus as described above. Four days following virus administration, mice were given an intra-peritoneal injection of either 3 mCi ^{131}I or saline (control group). All radioisotope handling was carried out under the guidance of the Radiation Safety Department at the Mayo Clinic. Intraperitoneal administration of radioiodide permitted rapid diffusion of the iodide while preventing leakage/spill of radioisotope that may occur using other routes. The volume of tumors infected with Ad/MUC1/NIS, Ad/CMV/NIS and control virus were monitored and compared on a weekly basis following administration of radioiodide, in both groups treated with ^{131}I, and those given saline. Tumors were measured using calipers, and volume estimated according to the formula: tumor volume = length x width x height x 0.52. Statistical significance was determined using the Students *t* test.

Results

***In Vitro* Iodide Uptake.** At varying time points following infection with Ad/CMV/NIS or Ad/MUC1/NIS, iodide uptake assays were performed as an indicator of NIS function. Optimal uptake of iodide in the MUC1 positive Capan-2 cells was observed four days following infection (Fig. 1), with essentially equal levels observed after infection with either construct, resulting in a 43-fold increase over control cells. In all cases, iodide uptake was blocked in the presence of $KClO_4$, a known inhibitor of NIS function.

***In Vitro* Iodide Efflux.** The rate of iodide efflux from Capan-2 cells was also determined as described by Weiss et al. (1984), at five minute intervals over the course of an hour (Fig. 2). Within the first 5 mins, 47% of concentrated iodide had been eluted, with 7% remaining at the 1 hr time point. The $t_{1/2}$ was calculated to be 5.8 mins.

***In Vivo* ^{123}I imaging.** Established subcutaneous Capan-2 tumor xenografts that were $\geq$100 mm^3 in volume were injected (intratumoral) with virus, followed four days later by an intraperitoneal injection of 0.5 mCi ^{123}I and serial image acquisitions on a γ-camera. In all cases, iodide uptake was observed at the site of the thyroid, salivary glands and stomach as a result of native NIS expression, and also at the site of the bladder where ^{123}I was being excreted (Fig. 3, taken 5 hrs following radioiodide injection). There was also a robust image of the Capan-2 tumors that had been infected with the MUC1/NIS construct. No iodide uptake was observed in tumors infected with control (promoterless) virus, or at any unexpected sites within the animals.

Although the replication deficient constructs used are intended only for intratumoral delivery, in order to address the possibility of virus leakage upon injection, IV administration of virus was also performed to assess the potential for hepatotoxicity. When the MUC1/NIS construct was used, no change in biodistribution of iodide was observed compared to non-injected animals (Fig. 4A). However, following systemic administration of the non-specific CMV/NIS construct, robust iodide uptake was observed throughout the mouse liver (Fig. 4B), with over 70 % of injected iodide concentrated in this area within one hour.

As planar images were performed, it was not possible to reliably separate the images of the mouse stomach from that of the liver. To address this, the liver was excised from animals that had received IV injection of virus, and total radioiodide counts performed using a γ-counter to confirm the absence of iodide in the livers of MUC1/NIS infected animals. The level of iodide in the livers of mice infected with CMV/NIS was ~50 fold higher than that detected in tissue from MUC1/NIS infected animals (Fig. 4C).

***In Vivo* ^{123}I efflux.** The serial images acquired permitted investigation of the rate of ^{123}I efflux from the MUC1/NIS-transduced tumors (Fig. 5). The percentage of the total dose administered that was concentrated at the tumor site was determined, and corrected for background, acquisition time and iodide decay (^{123}I half-life, 13.2 hrs). The average tumor activity per gram of tissue at 1, 3, 5 and 7 hrs was 10%, 11%, 13% and 12% respectively. Peak iodide uptake was observed approximately 5 hrs following iodide administration (13%) and had decreased to 6.5% of the total dose at 24 hrs. The final

images were acquired at 48 hrs, at which time there was no significant tumor image visible.

***In Vivo* ^{131}I Therapy.** Four days following intratumoral injection of MUC1/NIS (n=6), CMV/NIS (n=6) or control virus (n=6), mice were given an intraperitoneal dose of ^{131}I. Tumor volumes (L x W x 0.51) for each group were then recorded on a weekly basis and compared (Figure 6). There was a marked regression of tumors injected with both MUC1/NIS or CMV/NIS followed by ^{131}I therapy, while control tumors continued to increase in size. At five weeks following therapy, MUC1/NIS infected tumors had regressed to <70% of starting volume, and were further reduced to <50 % by week 10, in contrast to control tumors which had increased to >280 % of original volume by that time. CMV/NIS infected tumors also responded to ^{131}I therapy, with a mean 40 % reduction in tumor volume by week 5. Although treated tumors did eventually begin to grow again, they still remained significantly smaller than control tumors at 14 weeks following therapy ($P<0.0001$) with mean volumes of 64% (MUC1/NIS) and 126% (CMV/NIS) versus 427% (control).

Discussion

Standard therapy for pancreatic cancer usually involves surgery in combination with radiation and chemotherapy. If the disease is truly limited to the pancreas, it may be resectable, but >80% of patients present with extensive metastatic disease, for which there is no effective therapy (Moniaux *et al.*, 2004). Two of the most widely used serum markers for monitoring pancreatic cancer progression recognize epitopes carried by MUC1 (Hollingsworth *et al.*, 2004; Moniaux *et al.*, 2004). Both glycosylation and expression of MUC1 are deregulated during the development and progression of pancreatic cancer. Reports have shown a large increase in expression in tumor cells, with a loss of apical distribution, and expression throughout the tumor mass and on the surface of tumor cells (Mukherjee *et al.*, 2000; Levi *et al.*, 2004; Moniaux *et al.*, 2004). This change in profile of expression makes it cancer specific.

In this study we successfully introduced MUC1-driven NIS expression in pancreatic tumors to facilitate uptake and retention of iodide for radioiodide imaging and therapy of the disease. For adenovirus-mediated gene therapy to be successful cells must express high levels of the coxsackie adenovirus receptor (CAR) and this has been shown to be true of pancreatic tissue, providing for good transduction efficiency (Tomko *et al.*, 1997; Bergelson *et al.*, 1998). *In vitro* studies revealed a 43-fold increase in iodide uptake following infection with the MUC1/NIS construct. Remarkably, the levels of uptake were similar to those achieved using the potent non-specific viral CMV promoter. Specificity of the construct for MUC1 positive cells *in vitro* was shown in a previous report from this laboratory using a range of MUC1 positive/negative cell lines (Dwyer *et al.*, 2005a).

On the basis of the encouraging uptake levels *in vitro*, the work was translated to an *in vivo* setting. The use of NIS as a therapeutic gene provides for non-invasive imaging to assess the accuracy of gene delivery before proceeding to therapy. Mice with established subcutaneous pancreatic tumors received intratumoral injections of virus followed by intraperitoneal ^{123}I four days later. Following infection with MUC1/NIS, the Capan-2 tumors accumulated and retained iodide effectively. Robust images of the NIS-transduced tumors were seen with ~10% of administered iodide accumulated at the tumor site within one hour. No other change in iodide biodistribution was seen following infection. Images were also seen of the mouse thyroid and stomach due to native NIS expression, and at the site of the bladder as a result of iodide excretion. The level of gastric uptake in murine models is considerably higher than that seen in humans, where gastric side effects of radioiodide therapy are rare and mild (MIRD, 1975; Mazzaferri and Kloos, 2001). Also, thyroidal uptake of iodide can be effectively reduced in patients through the use of T_3 supplementation and TSH suppression (Mazzaferri and Kloos, 2001; Wapnir *et al.*, 2004).

The rate of iodide efflux from pancreatic cells *in vitro* was relatively fast (t½ = 5.8mins), and as anticipated, was considerably slower in 3D tumors *in vivo* compared to the monolayer culture (Carlin *et al.* 2000). In the animal model, peak uptake (13% of injected dose) was seen 5 hrs after iodide administration, with 6% still remaining 24 hrs following injection.

The use of a replication deficient adenovirus is a potential limitation of this system as it can not spread to neighboring cells or target metastatic disease. However, this limitation is also beneficial in terms of virus safety, allowing more complete control over the dose

administered. Also, due to the pathlength of the beta particles released during ^{131}I decay, there is a bystander effect, meaning that all target cells do not need to be transduced with NIS for an effect to be seen (Dingli *et al.*, 2003).

While intended for intratumoral injection, in order to address the potential for virus leakage and consequent hepatotoxicity, IV administration of adenovirus was performed. This assessed the presence of any non-specific promoter activity. *In vivo*, the liver is known to be the major organ for adenovirus clearance in mice (Reynolds *et al.*, 1999; Tao *et al.*, 2001). Images taken following IV CMV/NIS administration showed robust iodide uptake throughout the mouse liver/stomach area, with >70% of the injected dose of iodide concentrated in this region within two hours. IV injection of MUC1/NIS did not result in any apparent iodide uptake in the liver, demonstrating the selectivity, and potential safety of this construct. Since planar imaging was performed it was not possible to reliably separate the liver and stomach images, so the animals were sacrificed and total counts performed on liver sections in a γ-counter. This revealed a ~50-fold increase in iodide uptake in the CMV/NIS infected liver compared to its MUC1/NIS infected counterpart. These results demonstrate that the MUC1 promoter confers selective expression of NIS, and no hepatotoxicity would be expected even in the event of virus leakage. Further ongoing investigations, including detailed biodistribution and toxicity studies, are necessary to completely evaluate the safety of this construct.

Following assessment of the biodistribution of NIS expression through imaging, mice were given a therapeutic dose of ^{131}I. Within a few weeks of radioiodide therapy, NIS-transduced tumors showed a marked regression in volume. Tumors infected with MUC1/NIS continued to decrease in size reaching <50% of their starting volume within

10 weeks of treatment. Although tumors did eventually begin to grow again, even at 14 weeks following treatment, NIS-transduced tumors were less than ¼ the size of control tumors [$P<0.001$], which highlights the high proliferative rate of pancreatic tumors.

A recent study by Shen et al. (2004) reported the importance of radioiodide dose and intervention time on the outcome of ^{131}I therapy of tumors. Increasing the radioiodide dose may extend the effects seen in the current model. Also, the use of repeated injection/dosing may be beneficial. Although the use of adenoviral constructs does raise the issue of immunogenicity which would generally preclude repeat virus injection, the intratumoral route of delivery used here may reduce the immune response and thus permit repeat virus injection.

The data presented here provide strong evidence of the potency and selectivity of the MUC1/NIS construct in pancreatic cancer cells. There is currently no uniformly effective therapy available for metastatic pancreatic cancer, and survival has not improved significantly over the past 25 yrs (Jemal *et al.*, 2005). It is a highly aggressive disease with an average survival of 4-6 mths, and an increasing incidence worldwide. The ability to target NIS expression to pancreatic cancer, which has such limited treatment options, may be highly beneficial and warrants further investigation.

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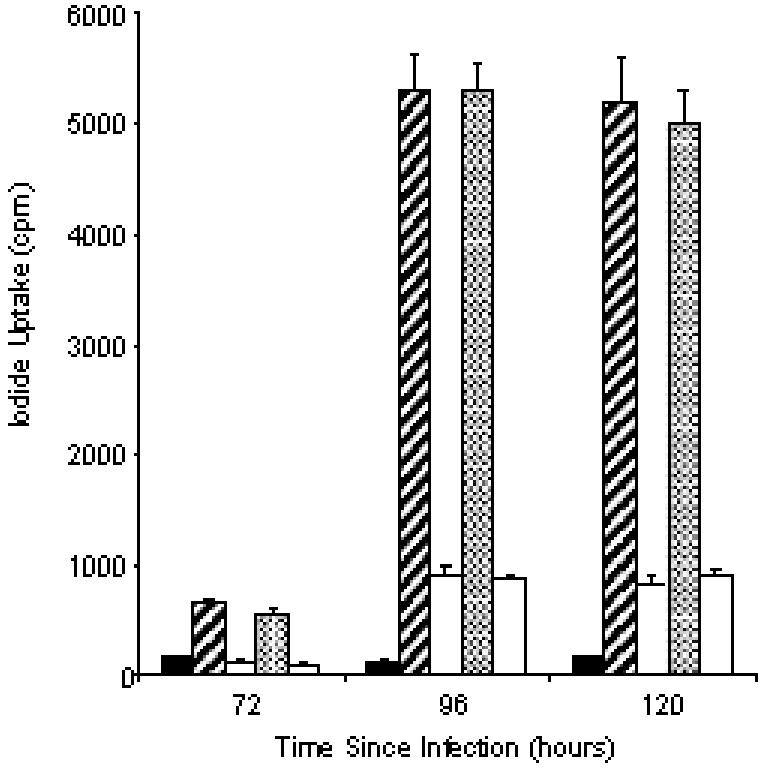

FIG. 1. Iodide uptake in Capan-2 cells at various timepoints following infection with Ad5/CMV/NIS (▨), Ad5/MUC1/NIS (⊡) or control (promoterless) (■) virus. Parallel iodide uptake assays were performed in the presence of $KClO_4$ (□), a known inhibitor of NIS function. The data presented represent mean ± SEM of triplicates.

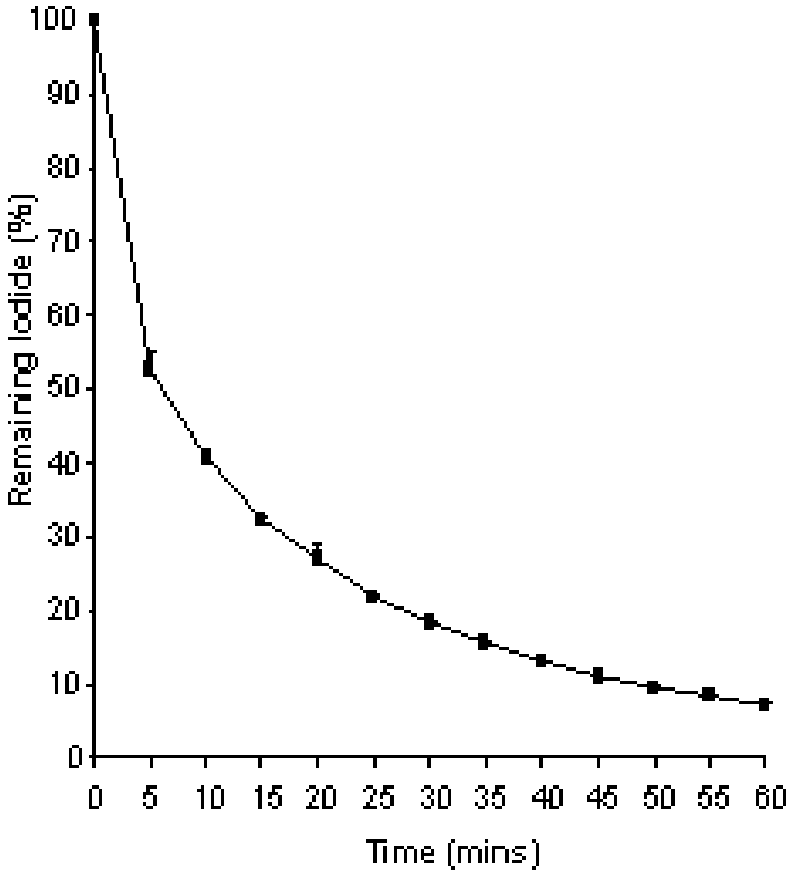

FIG. 2. Rate of iodide efflux from NIS-transduced Capan-2 cells.

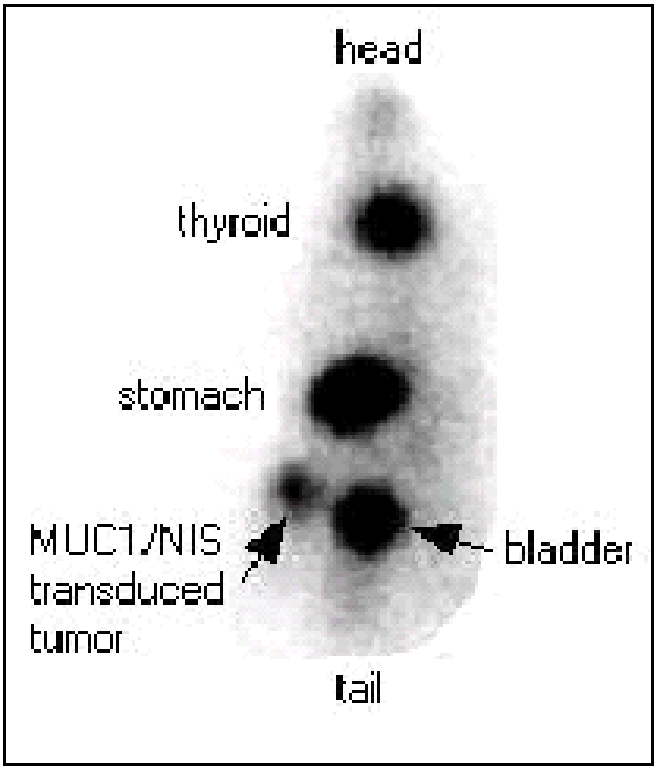

FIG. 3. In vivo imaging of ^{123}I distribution in a mouse model four days following intratumoral injection of Ad5/MUC1/NIS. Mice received an intraperitoneal dose of 0.5mCi ^{123}I followed by serial image acquisition on a γ-camera. Image shown was taken 5 hr after radioiodide administration.

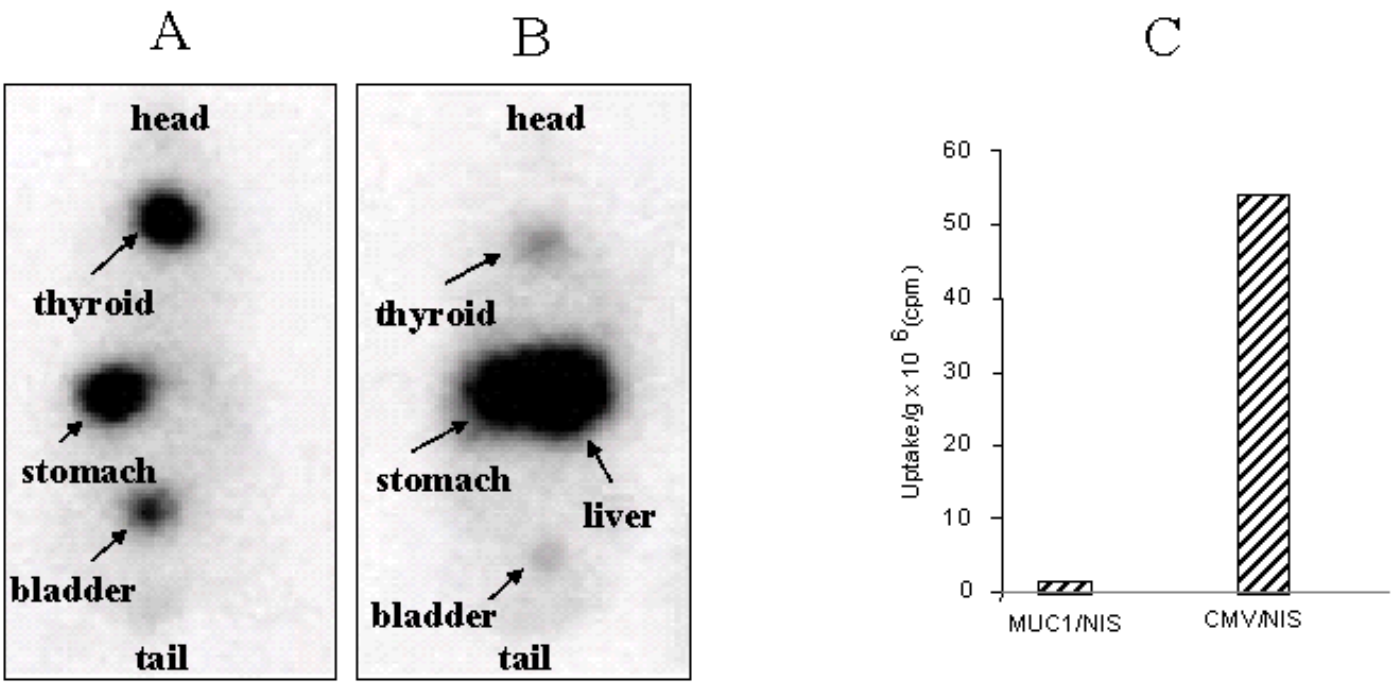

FIG. 4. In vivo imaging of ^{123}I distribution in a mouse model following intravenous administration of (**A**) Ad5/MUC1/NIS or (**B**) Ad5/CMV/NIS with intraperitoneal administration of 0.5mCi ^{123}I four days later. mages shown were taken 5 hr after radioiodide administration. After image acquisition, mouse livers were excised and counts of total radioiodide were performed with a counter (**C**).

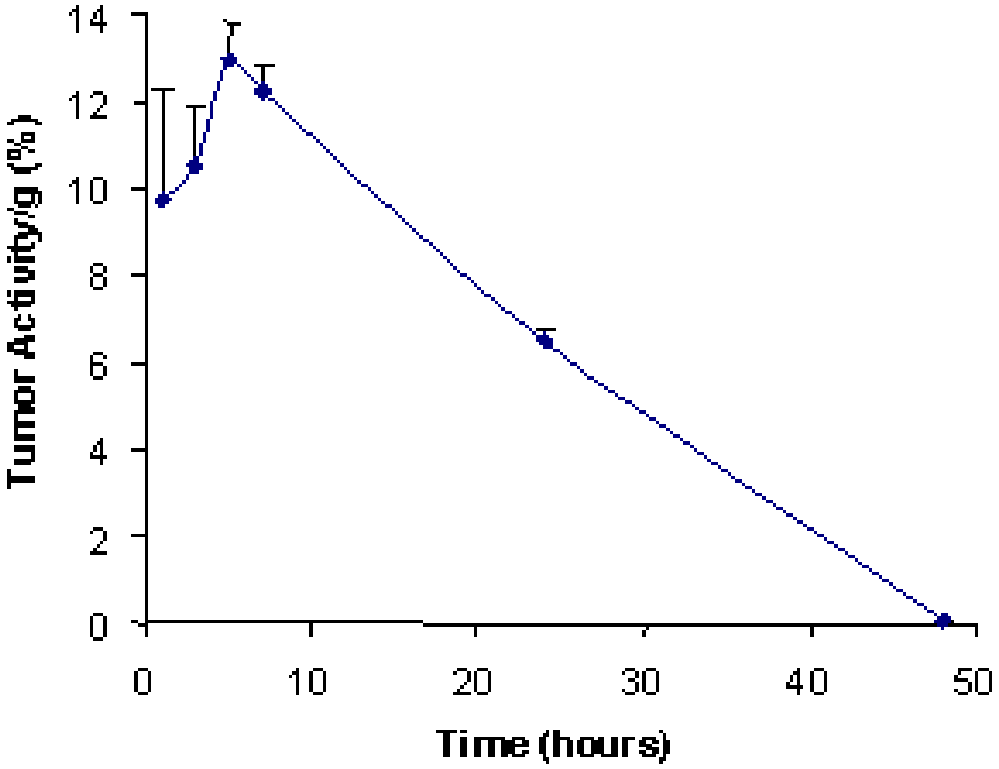

FIG. 5. *In vivo* 123I efflux from Capan-2 tumors. Tumors were infected with Ad5/MUC1/NIS followed by an intraperitoneal dose of 0.5 mCi of 123I 4 days later. Serial image acquisition permitted tracking of tumor iodide levels over a 48-hr period. Results were corrected for background, acquisition time, and iodide decay, and expressed as a percentage of the total dose administered.

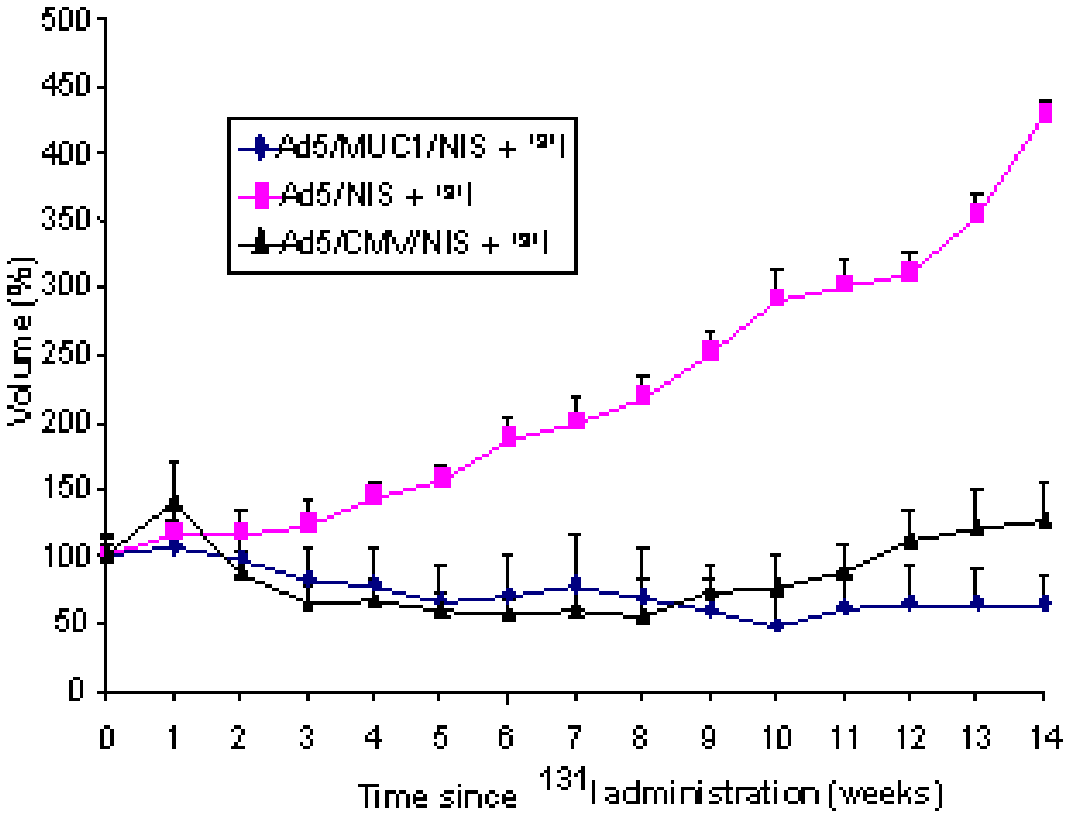

FIG. 6. *In vivo* 131I therapy of Capan-2 tumor xenografts. Four days after intratumoral injection of Ad5/MUC1/NIS virus (diamonds), Ad5/CMV/NIS virus (triangles), or control virus (promoterless; squares), mice were given an intraperitoneal dose of 131I. Tumor volumes (length _ width _ height _ 0.52) for each group were then recorded on a weekly basis, using calipers. Results are expressed as percentage of original tumor volume (mean _ SEM).