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Gene expression imaging with radiolabeled peptides

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An approach to image radiolabeled peptide localization at tumor sites by inducing tumor cells to synthesize membrane expressed human somatostatin receptor subtype 2 (hSSTr2) with a high affinity for radiolabeled somatostatin analogues is described. The use of gene transfer technology to induce expression of high affinity membrane hSSTr2 can enhance the specificity and degree of radiolabeled peptide localization in tumors. Employing this strategy, induction of high levels of hSSTr2 expression with selective tumor uptake of radiolabeled peptides was achieved in both subcutaneous non-small cell lung cancer and intraperitoneal ovarian cancer mouse human tumor xenograft models. The features of this genetic transduction imaging approach are: (1) constitutive expression of a tumor-associated receptor is not required; (2) tumor cells are altered to express a new target receptor or increased quantities of a constitutive receptor at levels which may significantly increase tumor targeting of radiolabeled peptides compared to uptake in normal tissues; (3) gene transfer can be accomplished by local or regional injection of adenoviral vectors; (4) it is feasible to target adenovirus vectors to tumor cells by modifying adenoviral tropism (binding) or by the use of tumor-specific promoters such that the hSSTr2 will be specifically expressed in the desired tumor; and (5) this technique can be used to image expression of a second therapeutic gene.

Key words: somatostatin receptor, peptide imaging, gene expression imaging

INTRODUCTION

THE GROUP of somatostatin receptors includes gene products encoded by 5 separate somatostatin receptor genes.[1] Human somatostatin receptor subtype 2 (hSSTr2) is expressed on a number of human tumors including neuroendocrine, ovarian, renal, breast, prostate, lung, and meningiomas.[2–6] The receptors have varying tissue levels in the brain, gastrointestinal tract, pancreas, kidney, and spleen.[7–9] All 5 receptors show high affinity binding to natural somatostatin peptide (either somatostatin-14 or somatostatin-28). Octreotide, P829, and P2045 are synthetic somatostatin analogues that preferentially bind with high affinity to somatostatin receptor subtypes 2, 3, and 5.[7–12]

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Octreotide is an eight amino-acid peptide which has a high affinity for hSSTr2, which is the most prominent somatostatin receptor on human tumors, and is stable toward *in vivo* degradation relative to the endogenous 14 amino-acid somatostatin-14 peptide. Octreotide and other somatostatin analogues have been modified with bifunctional chelating agents, for complexing radiometals, and by changing the amino acid sequence in order to increase their hSSTr2 binding affinity and optimize their normal organ clearance. Somatostatin analogues have been labeled with ^{111}In and ^{99m}Tc for imaging applications.

Radiolabeled peptides (*e.g.* ^{111}In-DTPA-D-Phe^1-octreotide, Octreoscan® or ^{99m}Tc-P829, Neotect®) have been used to target hSSTr2 positive tumor cells for imaging in patients.[13–16] Despite the success of these investigations, improved targeting strategies are possible using a genetic approach, in which a gene transfer vector is injected to express hSSTr2 on the tumor cell surface which can be imaged after injection of a radiolabeled peptide (Fig. 1). This approach also offers the possibility for imaging gene transfer of a second therapeutic gene. Our focus has been the development of recombinant

adenoviral vectors that transfer the hSSTr2 gene alone or with a second therapeutic gene to tumor cells and use of radiolabeled peptides to image gene transfer.[17–23] A potential advantage of genetic transduction of hSSTr2 is that the level of expression may be greater than what are generally otherwise low tumor concentrations of this receptor.

In this genetic approach we are attempting to specifically increase the number of receptors on tumor cells that normally express a receptor, or to specifically induce expression on tumor cells that do not ordinarily express the receptor by the use of genetic transduction. It is our hypothesis that one can deliver a larger fraction of the administered dose of the radiolabeled peptide to the tumor cells through increased receptor expression at the tumor site. The potential advantages of the genetic transduction approach are: (1) constitutive expression of a tumor-associated receptor is not required; and (2) tumor cells are altered to express a target receptor at levels which may significantly improve tumor-to-normal tissue targeting of radiolabeled peptides. This method thus represents a new paradigm by which imaging of gene transfer can be achieved through radiolabeled peptide localization to tumors transduced *in situ* to express unique and novel receptors.

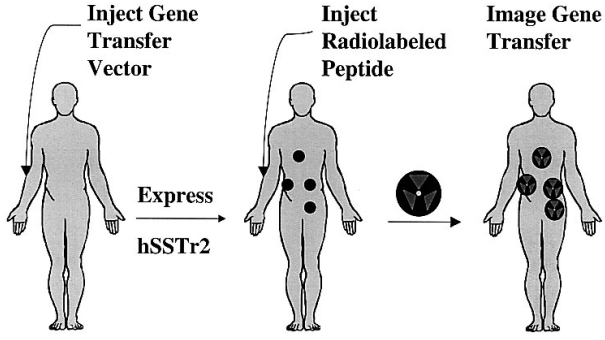

Fig. 1 Genetic radioisotope targeting strategy to image gene transfer.

GENETIC INDUCTION OF hSSTr2 *IN VITRO* AND *IN VIVO*

Imaging of human non-small cell lung and prostate cancer cells and tumor xenografts induced to express hSSTr2

An adenovirus encoding the gene for hSSTr2 under control of the cytomegalovirus promoter (AdCMVhSSTr2) was produced.[19] *In vitro* binding of ^{111}In-DTPA-D-Phe^1-octreotide to A-427 human non-small cell lung cancer cells infected with AdCMVhSSTr2 was demonstrated by imaging of cells in plates (Fig. 2).[24] Scatchard analysis of A-427 cells infected with 10 plaque forming units (pfu) AdCMVhSSTr2 and binding of ^{99m}Tc-P829 (Neotect) showed a B_{max} of 19,000 fmol/mg and the affinity of ^{99m}Tc-P829 to be 7 nM.[20]

Other studies showed the localization of ^{111}In-DTPA-D-Phe^1-octreotide to *s.c.* A-427 non-small cell lung tumors injected intratumorally (*i.t.*) with AdCMVhSSTr2.[24] The gamma camera region of interest analysis showed the tumor uptake of ^{111}In-DTPA-D-Phe^1-octreotide to be 2.8% ID/g 48 h after a single AdCMVhSSTr2 injection and 3.1% ID/g at 96 h (Fig. 3). Uptake of ^{111}In-DTPA-D-Phe^1-octreotide in control adenoviral-injected tumors with the thyrotropin-releasing hormone receptor gene (AdCMVTRHr) was <0.3% ID/g at both time points.

A novel ^{99m}Tc-labeled peptide (P2045) described by Diatide, Inc. binds with high affinity to hSSTr2.[11] We

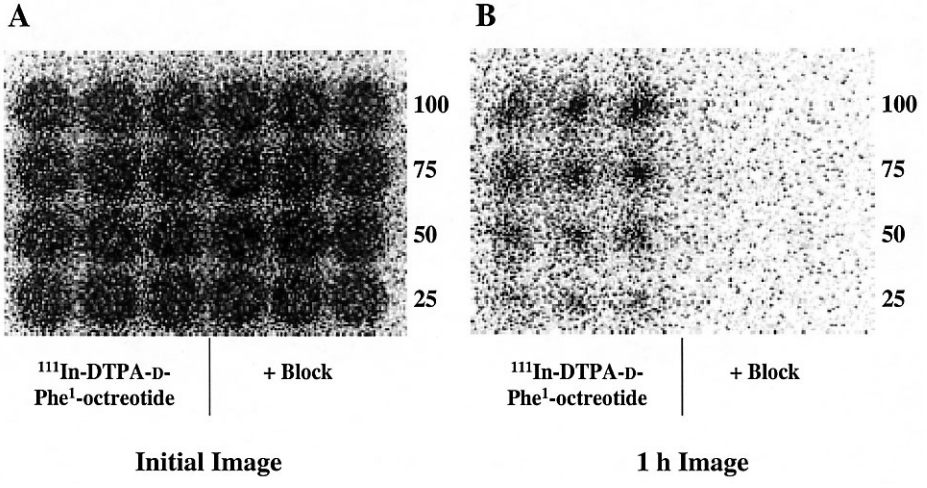

Fig. 2 Gamma camera imaging of AdCMVhSSTr2-infected A-427 non-small cell lung cancer cells mixed with uninfected cells (percentages of hSSTr2-positive cells indicated on the right) in a 24-well plate incubated with ^{111}In-DTPA-D-Phe^1-octreotide in the presence or absence of excess unlabeled octreotide as a blocking agent.[24] Reprinted with permission from the American Cancer Society.

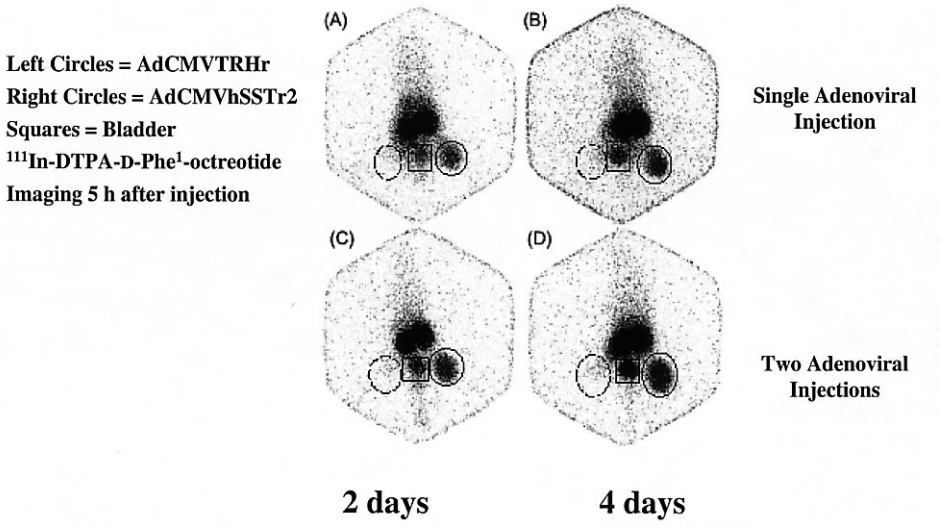

Fig. 3 Gamma camera images of mice after intratumoral AdCMVhSSTr2 injection into athymic nude mice bearing A-427 xenografts obtained at 2 or 4 days after *i.v.* injection of ^{111}In-DTPA-D-Phe^{1}-octreotide.[24] Reprinted with permission from the American Cancer Society.

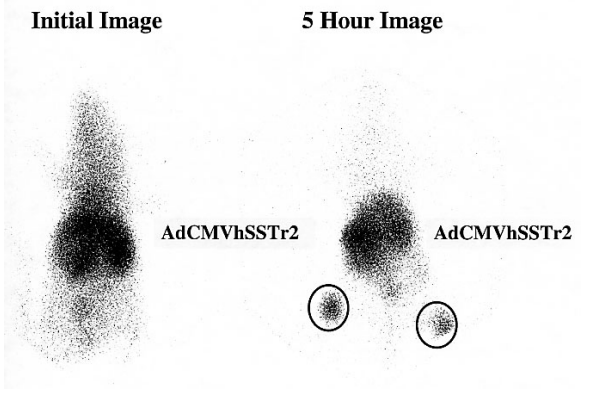

Fig. 4 Gamma camera imaging of ^{99m}Tc-P2045 in mice bearing DU145 *s.c.* tumor xenografts injected with AdCMVhSSTr2.

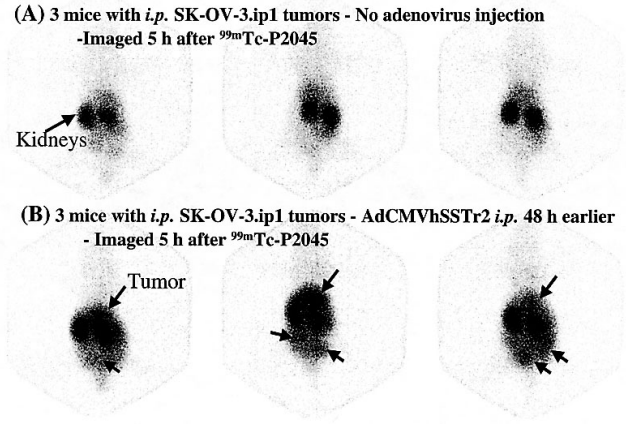

Fig. 5 Gamma camera imaging of AdCMVhSSTr2 gene transfer to *i.p.* SK-OV-3.ipl ovarian tumors after *i.v.* injection of ^{99m}Tc-P2045.[21] Reprinted with permission from Springer-Verlag.

evaluated P2045 in mice bearing *s.c.* A-427 tumors injected *i.t.* with AdCMVhSSTr2 or with a control adenovirus. ^{99m}Tc-P2045 was injected *i.v.* 2 or 4 days after AdCMVhSSTr2 injection and the animals were imaged using a gamma camera equipped with a pinhole collimator 3.5–4.5 h later. The images showed higher radioactive uptake in the tumors, compared with ^{99m}Tc-P829, for tumors injected with AdCMVhSSTr2 but background uptake in tumors injected with control adenovirus. No other tissue had greater uptake than the AdCMVhSSTr2-injected tumor[25]; ~85% ^{99m}Tc-P2045 was excreted by 4 h.

Gamma camera imaging was also used to detect hSSTr2 expression in DU145 prostate tumor xenografts infected with AdCMVhSSTr2 using ^{99m}Tc-P2045. Mice bearing *s.c.* DU145 tumors injected *i.t.* with AdCMVhSSTr2 showed uptake of *i.v.*-injected ^{99m}Tc-P2045 detected by gamma camera imaging (Fig. 4), while uptake was not observed when the tumors were infected with a control adenovirus (not shown). Uptake averaged 7.5 ± 1.0% for AdCMVhSSTr2 injected tumors. Of note, the tumor sizes for the mouse in Figure 4 were 144 and 167 mg for the left and right tumors, respectively. Independent confirmation of hSSTr2 expression was demonstrated by immunohistochemical analysis.

Imaging of human ovarian cancer xenografts induced to express hSSTr2

To evaluate the ability to induce receptor expression in ovarian cancer *in vivo*, AdCMVhSSTr2 was injected *i.p.* to induce hSSTr2 expression on SK-OV-3.ipl human ovarian tumors 5 days after tumor cell injection in the peritoneum in athymic nude mice. Two days later, tumor localization of ^{111}In-DTPA-D-Phe^{1}-octreotide at 4 h after *i.p.* injection was equal to 60.4% ID/g.[19] The uptake in

tumor decreased to 18.6% ID/g at 24 h after injection. The tumor localization was significantly lower (1.6% ID/g) when a control adenovirus encoding the gene for gastrin releasing peptide receptor (AdCMVGRPr) was injected. Imaging of hSSTr2 gene transfer to *i.p.* SK-OV-3.ipl tumors after injection of ^{99m}Tc-P2045 is shown in Figure 5.[21] Thus, these studies demonstrated that tumor uptake of ^{111}In-DTPA-D-Phe^{1}-octreotide and ^{99m}Tc-P2045 could be detected after infection of the ovarian tumors *in vivo* with AdCMVhSSTr2.

Tumor uptake of ^{99m}Tc-P2045 in nude mice bearing SK-OV-3.ipl tumors in the peritoneum at 48 h after *i.v.* injection averaged 2.2 ± 0.3% ID/g for mice injected *i.p.* with AdCMVhSSTr2 (1×10^9 pfu) as compared to 0.2 ± 0.002% ID/g in control mice not receiving adenoviral injection ($p < 0.05$) or in tumors from mice injected *i.p.* with an adenovirus encoding the green fluorescent protein (AdCMVGFP) which averaged 0.3 ± 0.2% ID/g.[26]

IMAGING OF THERAPEUTIC GENE EXPRESSION

The molecular suicide gene therapy approach involves insertion and expression of an enzyme in a target cell that converts a non-toxic prodrug to a toxic drug.[27,28] The two most widely studied systems are thymidine kinase (TK) and cytosine deaminase (CD). The enzyme CD is a non-mammalian enzyme which catalyzes the formation of uracil by the deamination of cytosine. When 5-fluorocytosine (5-FC) is the substrate, this enzyme will produce 5-fluorouracil (5-FU), a potent cancer chemotherapeutic and radiosensitizing agent.[29] The genes for bacterial and yeast CD have been cloned.[30–33] Because mammalian cells do not normally express the CD gene, 5-FC is nontoxic to these cells, even at high concentrations. 5-FC has been used as an antifungal drug because of its relative nontoxicity in humans. The CD gene has been used in gene therapy strategies to mediate intracellular conversion of 5-FC to 5-FU, and has been shown to be effective in animal tumor models.[34,35] This therapeutic strategy has the advantage of intracellular production of high concentrations of radiosensitizing drug as an alternative to systemic administration, therefore potentially limiting systemic toxicities.[36] Converted 5-FU passively diffuses across the cell membrane from CD-positive cells to nontransduced cells.[37,38] This bystander effect compensates for the inability of current vector systems to transduce all but a small fraction of cells in a given tumor.[28,39] The use of adenoviral vectors to encode CD and convert 5-FC to 5-FU to achieve cell killing has been reported by our group[40–43] and others.[35,38,44,45] We initially used an adenoviral vector encoding CD under control of the cytomegalovirus promoter (AdCMVCD) in combination with 5-FC and radiation treatment to demonstrate enhanced cytotoxicity against human colon, pancreatic, glioblastoma, and cholangiocarcinoma cells *in vitro* and *in vivo*.[40–42] More recently, we have used two gene vectors in which hSSTr2 has been used as an imaging gene and CD has been used as a therapeutic gene.

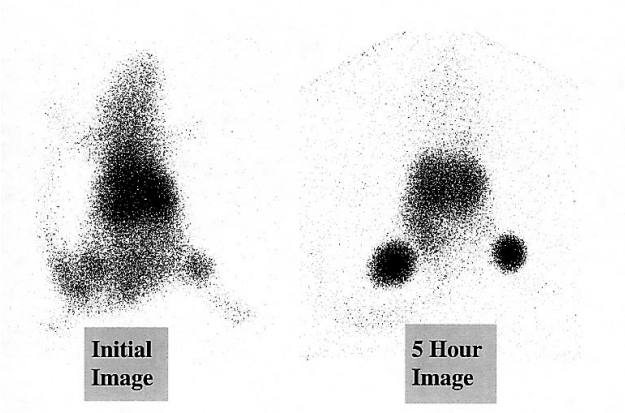

Fig. 6 Imaging of gene transfer in mice bearing A-427 *s.c.* tumors injected with AdCMVhSSTr2CD (*left*) or AdRGDCMVhSSTr2CD (*right*) followed 2 days later by *i.v.* injection of ^{99m}Tc-P2045.

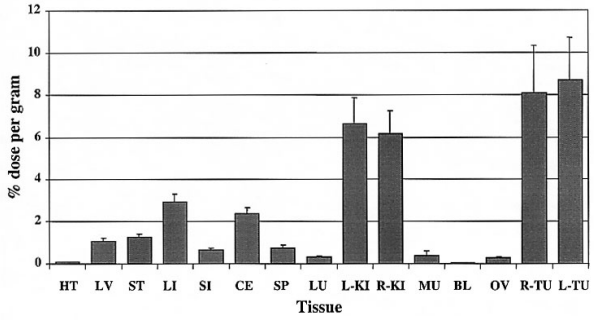

Fig. 7 Biodistribution of ^{99m}Tc-P2045 at 5 h after *i.v.* injection in nude mice bearing *s.c.* A-427 tumors injected with AdCMVhSSTr2CD (R-TU) or AdRGDCMVhSSTr2CD (L-TU). HT, heart; LV, liver; ST, stomach; LI, large intestine; SI, small intestine; CE, cecum; SP, spleen; LU, lungs; L-KI, left kidney; R-KI, right kidney; MU, leg muscle; BL, blood; OV, ovary; R-TU, right tumor; L-TU, left tumor.

Imaging of cytosine deaminase therapeutic gene transfer

Bicistronic adenoviral vectors encoding for hSSTr2 and the CD enzyme have been constructed and evaluated.[46] One rationale for the construction of these vectors is that hSSTr2 can be used as a target for non-invasive imaging to determine the expression of the therapeutic gene (CD) *in vivo*.[23] Second, hSSTr2 can be used for radiolabeled peptide therapy and the combination of this with CD mediated therapy through conversion of 5-FC to 5-FU may result in an additive or synergistic therapeutic effect. A-427 cells infected with bicistronic vectors AdCMVhSSTr2CD or AdRGDCMVhSSTr2CD, with the RGD peptide genetically engineered in the fiber knob to retarget adenovirus binding to integrins on the cell

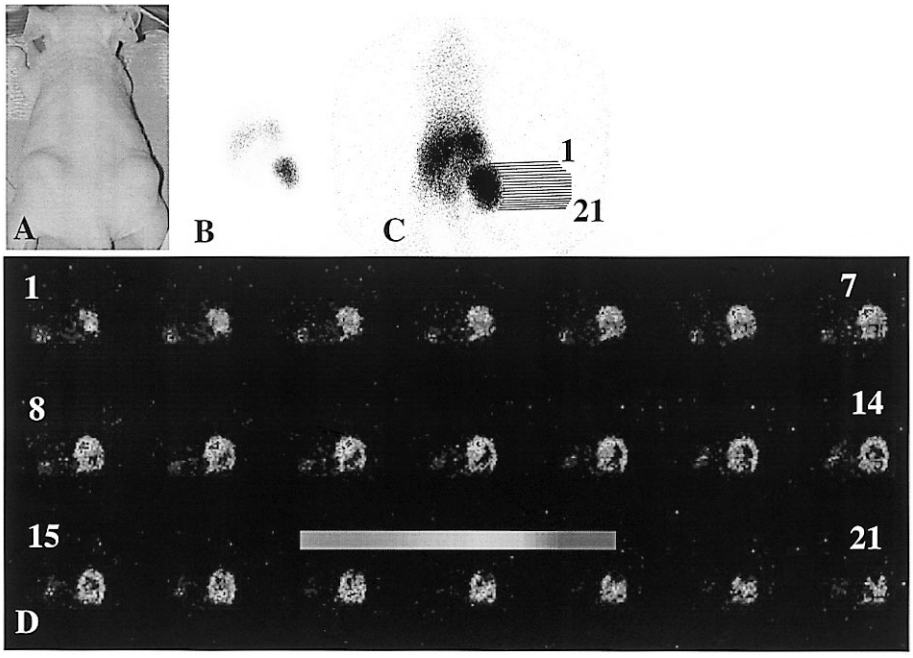

Fig. 8 SPECT imaging of adenovirus-mediated hSSTr2 expression in a tumor xenograft. (A) Photograph of representative mouse with two A-427 tumors. The right tumor was injected with 1×10^9 pfu AdhSSTr2GFP while the left tumor was injected with a control adenovirus vector. (B, C) planar gamma camera imaging at 5 h after *i.v.* ^{99m}Tc-P2045 injection, (D) Transverse slices (0.58 mm each) showing hSSTr2 expression within the tumor. The adenovirus-mediated hSSTr2 expression led to specific retention of ^{99m}Tc-P2045 in discrete areas of the tumor. Figure 8B shows the highest retention of the ^{99m}Tc-P2045 was in the right tumor.

surface, produced equivalent hSSTr2 expression as the single gene vector AdCMVhSSTr2.[47] In addition, the AdCMVhSSTr2CD and AdRGDCMVhSSTr2CD vectors produced similar CD enzyme activity levels as the single gene vector AdCMVCD.[47] Thus, both genes were active in the bicistronic vectors. We next investigated imaging of gene transfer in athymic nude mice bearing *s.c.* A-427 tumors injected with 1×10^9 pfu AdCMVhSSTr2 (left) or AdRGDCMVhSSTr2CD (right). After 2 days, ^{99m}Tc-P2045 was *i.v.* injected and imaging was conducted (Fig. 6). The corresponding biodistribution results obtained by counting of tissues in a gamma camera are shown in Figure 7.

SPECT imaging was applied to measure the distribution of adenovirus-mediated transgene expression within *s.c.* xenografts. Nude mice bearing two A-427 flank tumors were injected *i.t.* with bicistronic adenovirus vectors. The bicistronic Ad5 vector encoding hSSTr2 and green fluorescent protein (GFP) was *i.t.* injected (1×10^9 pfu) in the right A-427 tumor, while a control bicistronic vector was injected in the left tumor. Imaging studies were conducted after 2 days. A photograph of a representative mouse with two tumors is shown in Figure 8A. Planar gamma camera images are presented in Figures 8B and 8C at 5 h after *i.v.* injection of ^{99m}Tc-P2045 (55.5 MBq). The two planar images are identical, with the exception of scaling; Figure 8C shows the location of transverse slices (0.58 mm each) that are displayed in Figure 8D. The distribution of hSSTr2 expression within the tumor was non-uniform and tended to be concentrated at the periphery of the tumor (Fig. 8D). The SPECT imaging technique had sufficient sensitivity and spatial resolution to enable the 3-dimensional hSSTr2 expression to be measured. The A-SPECT system (Gamma Medica, Inc., North Ridge, CA) was used for this study, with a total of 64 individual projections collected (30 sec each) using a 1 mm pinhole collimator.

Imaging of thymidine kinase therapeutic gene transfer

Further studies were carried out using a bicistronic adenoviral vector encoding hSSTr2 and TK in the A-427 tumor model.[23] The tumors were injected *i.t.* with the bicistronic vector (AdCMVhSSTr2TK) and the animals imaged for hSSTr2 expression with ^{99m}Tc-P2045 and TK with ^{131}I-FIAU (Fig. 9). The biodistribution results showed the uptake of ^{99m}Tc-P2045 and ^{131}I-FIAU for AdCMVhSSTr2TK-injected tumors (n = 8) was 11.1% and 1.6% ID/g, respectively. AdCMVhSSTr2-injected tumors (n = 4) accumulated 10.2% ID/g of the ^{99m}Tc-P2045 and 0.3% of the ^{131}I-FIAU. AdCMVTK-injected tumors (n = 4) had 0.2% ID/g for the ^{99m}Tc-P2045 and 3.7% for ^{131}I-FIAU.

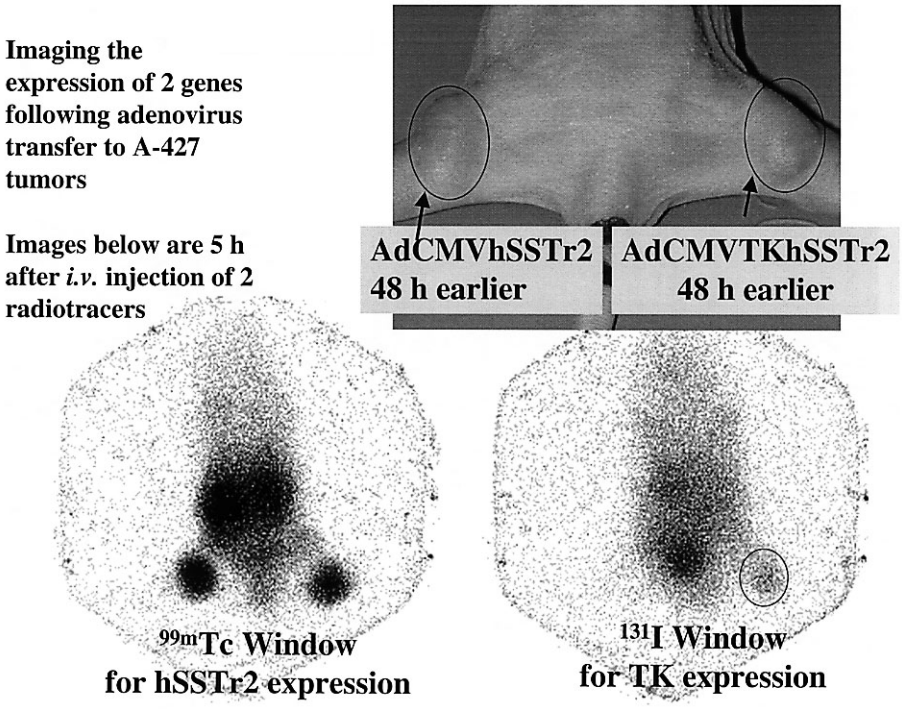

Fig. 9 Simultaneous imaging for hSSTr2 and TK expression in mice bearing *s.c.* A-427 tumors injected with AdCMVhSSTr2 or AdCMVTKhSSTr2. Photograph of the mouse shows tumor locations and adenoviral doses. The expression of hSSTr2 was identified by imaging tumor accumulation of ^{99m}Tc-P2045 (*bottom left*), while TK expression was depicted by imaging tumor accumulation of ^{131}I-FIAU (*bottom right*). The images were obtained 5 h after *i.v.* injection of the radiotracers.[23] Reprinted with permission from the Radiological Society of North America, Inc.

Table 1 Conversion of 5-FC to 5-FU in pmol/min/mg ± SD measured over a 1 h period after infection of DU145 cells with 100 pfu of each virus.

Vector	DU145
Uninfected	0.00
AdCMVCD	25.6 ± 1.3
AdCMVCDhSSTr2	7.5 ± 0.3
AdCox-2LCDhSSTr2	3.0 ± 0.2
AdCox-2LhSSTr2CD	2.5 ± 0.2

Table 2 Cytotoxicity of adenoviral infected DU145 human prostate cancer cells (10 pfu) exposed to 5-FC expressed as IC_{50} (nM).

Vector	DU145
Uninfected	202.6
AdCox-2LCDhSSTr2	13.1
AdCox-2LhSSTr2CD	21.4

Imaging of hSSTr2 gene transfer with adenoviral vectors controlled by the cyclooxygenase-2 promoter

The specificity of vectors for gene transfer may be improved by combining transductional targeting of adenovirus utilizing the tumor-specific promoter, cyclooxygenase-2 (Cox-2), to drive the transcription of the hSSTr2 and CD genes. The liver is the predominant site of adenovirus vector localization after systemic administration, and as a consequence is at risk when adenoviral vectors containing suicide genes such as CD ectopically localize to this site. Thus, a promoter with both tumor specificity and minimal transcriptional activity in hepatocytes would be ideal for cancer gene therapy.

Two new adenoviral vectors expressing CD and hSSTr2 were produced using the long (L) length Cox-2 promoter: AdCox-2LCDhSSTr2 and AdCox-2LhSSTr2CD. The new vectors expressing CD and hSSTr2 were tested to determine their conversion of 5-FC to 5-FU following infection of DU145 human prostate cancer cells (Table 1). The results indicate that AdCox-2LCDhSSTr2 and AdCox-2LhSSTr2CD produced a lower level of conversion of 5-FC to 5-FU in DU145 cells than AdCMVCD or AdCMVCDhSSTr2. These results support earlier observations that the CMV promoter is stronger than the Cox-2 promoter, although less specific.

In the next set of studies, we evaluated the cytotoxicity of the new vectors after infection of DU145 cells with 10 pfu/cell of virus. At 24 h after infection, the cells were seeded at 5,000 cells/well and exposed to varying concentrations of 5-FC. Cell viability was assessed by the MTS

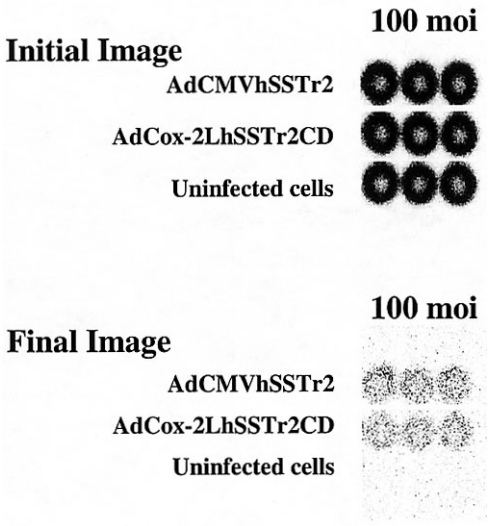

Fig. 10 Gamma camera imaging of hSSTr2 gene transfer to DU145 prostate cancer cells incubated with ^{99m}Tc-P2045 before (initial) and after (final) acid washing.

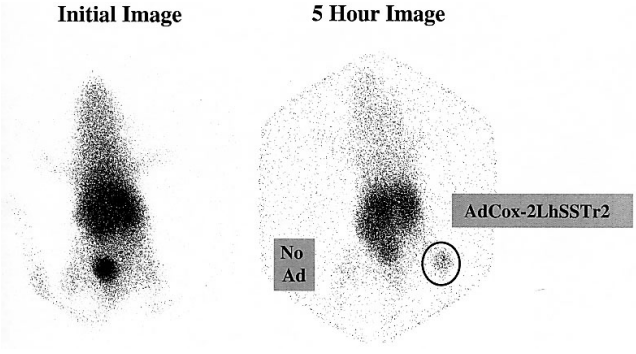

Fig. 11 Gamma camera imaging of ^{99m}Tc-P2045 administered *i.v.* in a nude mouse bearing DU145 *s.c.* tumors injected two days earlier with no adenovirus (Ad) or AdCox-2LhSSTr2.

assay (Celltiter 96 AQ, Promega). The IC_{50} values for these cell lines are shown in Table 2. The results indicate that AdCox-2LCDhSSTr2 and AdCox-2LhSSTr2CD produced cytotoxicity with a moderately low IC_{50} value.

In vitro binding of ^{99m}Tc-P2045 to DU145 cells infected with AdCMVhSSTr2 or AdCox-2LhSSTr2CD in plates detected by imaging with a gamma camera is shown in Figure 10. Gamma camera imaging of ^{99m}Tc-P2045 localization in a DU145 *s.c.* tumor injected with AdCox-2LhSSTr2 is shown in Figure 11. The combination of the therapeutic genes CD and hSSTr2 with the Cox-2L promoter should provide specificity for tumor uptake of radiolabeled peptides that bind to hSSTr2 and selective 5-FC molecular chemotherapy. However, the level of hSSTr2 expression from AdCox-2LhSSTr2CD was lower than with AdCMVhSSTr2CD infection. Thus, we are currently producing RGD modified Cox-2L CD and hSSTr2 two-gene vectors to achieve higher levels of hSSTr2 and CD expression.

CONCLUSIONS

These studies demonstrate that genetic induction of hSSTr2 results in tumor localization of radiolabeled peptides at a level sufficient for imaging. It should be emphasized that in this approach we are attempting to specifically increase the number of receptors on tumor cells that normally express a receptor, or to specifically induce expression on tumor cells that do not ordinarily express the receptor by the use of genetic transduction, with the result being increased targeting of the radiolabeled peptide to the tumor site. The potential advantages of the genetic transduction approach are: (1) constitutive expression of a tumor-associated receptor is not required; and (2) tumor cells are altered to express a target receptor at levels which may significantly improve tumor-to-normal tissue targeting of radiolabeled peptides. This method thus represents a new paradigm by which imaging of gene transfer can be achieved through radiolabeled peptide localization to tumors transduced *in situ* to express unique and novel receptors. For approaching disseminated disease, transductional targeting approaches can be used to alter the tropism of viral vectors to achieve tumor cell specific gene delivery. Selective tumor targeting may also be achieved by transcriptional approaches employing tumor-specific regulatory elements. Other approaches that are under active investigation in other laboratories include gene transfer of the type-2 dopamine receptor expressed on the tumor cell surface detected by PET using a radiolabeled antagonist or herpes simplex virus TK gene transfer detected by gamma camera or PET imaging with radiolabeled substrates trapped intracellularly after phosphorylation by the kinase.[48–50] Gene transfer of the sodium iodide symporter has also been used for imaging with radioiodine.[51] Additional studies are needed to confirm the feasibility of these approaches in humans.

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