Prostate Cancer Specific Adenoviral Vectors to Increase the Therapeutic Index of Targeted Radiotherapy

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Introduction

The goal of this proposal was to construct and evaluate adenoviral vectors encoding for the human somatostatin receptor subtype 2 (SSTr2) in the context of human prostate cancer. It is hypothesized that these vectors can specifically increase SSTr2 expression on prostate cancer, which can then be targeted with the somatostatin analogue, ^{90}Y -SMT 487, for therapeutic purposes. Two of the major limitations of adenoviral vectors as gene delivery vehicles are that they lack selectivity for infecting target tissue (tumor) and that many cancers are refractory to adenoviral infection. To address the former issue adenoviral vectors will be constructed that contain SSTr2 driven by the cyclooxygenase-2 (COX-2) promoter. It has been shown that prostate cancers overexpress COX-2, thus construction of an adenoviral vector that contains a COX-2 promoter should limit SSTr2 expression to COX-2 positive tissues. The latter issue has been addressed by incorporating arginine-glycine-aspartic acid (RGD) residues into the adenoviral protein coat. Thus, the first specific aim of this proposal was: To construct two AdCOX-2SSTr2 vectors and evaluate them in human prostate cancer cells in vitro in comparison to the first generation AdCMVSSTr2 vector. Specifically, AdCOX-2SSTr2 and AdCOX-2SSTr2RGD was to be constructed and evaluated in PC-3, DU-145, and LNCaP human prostate cancer cells.

Materials and Methods

Construction of Adenoviral Vectors

AdCMVSSTr2 was produced and titrated against 293 human transformed embryonal kidney cells as previously described. The AdCOX-2SSTr2 was constructed through homologous recombination in *Escherichia coli* using the AdEasy system. The COX-2 promoter (-883/+59, SacI-HindIII fragment) was derived from pHES2 (provided by Drs. Inoue and Tanabe at National Cardiovascular Center Research Institute, Japan) and placed in front of SSTr2 for selective expression. Though three major control regions of the COX-2 promoter (binding sites of NF-kB, NFIL-6 and CRE) exist within 300 base pairs from transcription initiation site, the longer control regions were used to achieve as much fidelity as possible.

The AdCOX-2SSTr2RGD was made by configuring COX-2SSTr2 into the relevant shuttle vectors such that it was flanked with segments of Ad genomic DNA adjacent to the E1 region or to the fiber gene, respectively. The shuttle vector for the RGD replacement, pNEB.PK3.6 was designed previously. The recombinant gene construct assembled in the shuttle vector was transferred into the Ad genome contained within rescue vector pVK454 via homologous DNA recombination in *E. Coli*. The viruses were propagated in the adenovirus packaging cell line, 293, and purified by double CsCl density gradient centrifugation, followed by dialysis against phosphate-buffered saline with 10% glycerol. The vectors were titrated by plaque assay and stored at -80°C until usage.

Quantitation of SSTr2 expression after AdCOX-2SSTr2 infection

DOE Patent Clearance Granted

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DU-145 or LNCaP cells were infected with AdCMVSSTr2 or AdCOX-2SSTr2 at 100 plaque forming units (pfu) per cell. The cells were seeded such that they were ~80% confluent at the time they were infected and then harvested 2 days later for the membrane preparation. The membranes were prepared by rinsing the cells once with PBS, scraping them from the bottom of the flasks into PBS and centrifuging into a pellet. The pellets were then suspended in cold lysis buffer (10 mM Tris-Cl, pH 7.2, 2 mM EDTA, 2 mM MgCl₂, 0.5 mM PMSF) for 15 min, vortexed, centrifuged and the supernatant collected. This was repeated 4 times. The supernatants were centrifuged at 28,000 g for 30 min at 4°C. After centrifugation, the pellet was resuspended in 0.25 ml buffer (20 mM glycylglycine, 1 mM MgCl₂, 250 mM sucrose) and the protein concentration determined. Aliquots were frozen at -80°C. For the binding assay, 25 µg of the membrane preparation was washed twice and resuspended in 0.2 ml binding buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM MgCl₂, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 0.5 µg/ml aprotinin, 200 µg/ml bacitracin, and 0.1% BSA). ¹²⁵I-somatostatin (~0.01 nM) was added along with various concentrations (~0.01 - 100 nM) of unlabeled somatostatin to triplicates of the membrane preparation and incubated with shaking for 90 min at 25°C. After incubation, the membrane preparations were washed twice with ice cold wash buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM MgCl₂, 0.1% BSA), the supernatant aspirated, and the membrane pellet counted in a well-type gamma counter. The data was then plotted as a homologous competitive binding curve using the GraphPad Prism program.

In vivo expression of SSTr2 after AdCOX-2 infection.

Athymic nude mice were implanted s.c. with 1×10^7 DU-145 cells mixed 1:1 with Matrigel in each rear flank. Three weeks later the tumors were either left uninjected, or injected directly with 1×10^9 pfu of AdCMVSSTr2 or AdCOX-2SSTr2. Two days after adenoviral injection, the mice were imaged at 0 and 5 h after injection of ^{99m}Tc-P2045 (a SSTr2 binding peptide) using planar imaging techniques. During imaging procedures the animals were anesthetized with halothane gas anesthesia. The mice were imaged with an Anger gamma camera equipped with a pinhole collimator. Mice were positioned in ventral recumbancy with the legs extended from the body. For single image sessions, at least 50,000 total counts per image were collected. Images were processed on a Pegasys processing system using standard region of interest analysis. Regions of interest included tumor and whole animal. A background region was drawn outside the animal image to correct the whole-mouse and tumor regions of interest. Total counts and pixels were recorded for all regions. The average counts per pixel for the background region was multiplied by the pixels in the whole animal and tumor regions of interest; that number was then subtracted from the counts in each respective region to yield the actual count values for the whole animal and tumor. The fraction of activity in the tumor region was calculated as the ratio between the background corrected counts in the tumor region, divided by the initial (immediately after i.v. injection) background corrected counts in the whole animal. Immediately after the imaging session, the animals were sacrificed and the blood, lungs, liver, small intestine, spleen, kidney, bone, muscle, tumor, abdominal lining, uterus, and pancreas were removed, weighed, and counted in a gamma counter.

Results

Both AdCOX-2SSTr2 and AdCOX-2SSTr2RGD have been constructed and validated by RT-PCR. This is one of the deliverables for Specific Aim #1. The remaining results discuss the

evaluation of AdCOX-2SSTr2 in more detail in vitro and in vivo. AdCOX-2SSTr2RGD will be evaluated in more detail in the second year of the project.

Infection of DU-145 cells or LNCaP cells with AdCMVSSTr2 at 100 pfu/cell resulted in SSTr2 expression of $8,200 \pm 2,900$ fmol/mg of protein and $10,600 \pm 1,300$ fmol/mg, respectively. Infection of the COX-2 positive DU-145 cells with AdCOX-2SSTr2 resulted in SSTr2 expression of $2,600 \pm 2,200$ fmol/mg compared to $1,500 \pm 1,200$ fmol/mg for the COX-2 negative LNCaP cells. This represents 32% of expression of AdCMVSSTr2 for DU-145 cells and 14% of AdCMVSSTr2 for LNCaP cells.

DU-145 tumors could be visualized by gamma camera imaging after the tumors were injected with either AdCMVSSTr2 or AdCOX-2SSTr2, but not when the tumors were left uninjected. The tumor localization appeared to be more intense for tumors injected with AdCMVSSTr2 than with tumors injected with AdCOX-2SSTr2. Biodistribution of the animals confirmed this as 7.5% of the injected dose/ gram of tissue (ID/g) localized to AdCMVSSTr2 injected tumors compared to 0.75% ID/g for AdCOX-2SSTr2 injected tumors and 0.06% ID/g for uninjected tumors. The uptake in AdCOX-2SSTr2 injected tumors was comparable to the localization in other soft tissues.

Discussion

These studies show that both AdCOX-2SSTr2 and AdCOX-2SSTr2RGD have been produced. AdCOX-2SSTr2 was evaluated in DU-145 and LNCaP cells and showed that SSTr2 expression was 32% and 14% of AdCMVSSTr2, respectively. It was expected that SSTr2 expression would be higher in the COX-2 positive DU-145 cells and it was surprising that the SSTr2 expression was as high as it was in the LNCaP cells. This was partially confirmed in the DU-145 cells in vivo, where SSTr2 expression was only 10% of the expression after AdCMVSSTr2 infection. Although only 10% of AdCMVSSTr2, expression of SSTr2 after AdCOX-2SSTr2 infection was above background and could be visualized using a gamma camera. Also, the expression of SSTr2 in the liver will be investigated in future studies after i.v. injection AdCOX-2SSTr2 and AdCMVSSTr2. It is hypothesized that liver expression of SSTr2 will be lower after AdCOX-2SSTr2 infection compared to AdCMVSSTr2 infection. Thus, it may be possible to give higher doses of AdCOX-2SSTr2 to achieve expression of SSTr2 that is similar to that achieved with AdCMVSSTr2, yet without the toxicity associated with AdCMVSSTr2. The AdCOX-2SSTr2 will also be evaluated in the COX-2 positive PC-3 cells as originally proposed. Finally, the AdCOX-2SSTr2RGD will be evaluated to determine if this vector results in higher expression of SSTr2 in COX-2 positive cells. If all of these strategies fail, another approach will be investigated for treating prostate cancer xenografts which involves the use of an adenoviral vector encoding for both SSTr2 and the cytosine deaminase (CD) enzyme. It is hypothesized that this vector can increase the therapeutic efficacy of SSTr2-directed radiotherapy by CD converting the prodrug 5-fluorocytosine to the toxic and radiosensitizing drug 5-fluorouracil.