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MOLECULAR IMAGING OF GENE TRANSDUCTION IN CARDIAC TRANSPLANTATION

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by

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DEDICATION

I would like to dedicate this work to my parents for making great personal sacrifices to provide me with opportunities for personal development and career progression.

Molecular Imaging of Gene Transduction in Cardiac Transplantation

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ABBREVIATIONS USED IN THIS THESIS

Ad	Adenovirus
APC	Antigen Presenting Cell
CAR	Coxsackie virus and Adenovirus Receptor
CAV	Cardiac Allograft Vasculopathy
CEA	Carcinoembryonic Antigen
CD	Cluster of Differentiation (molecule)
CMV	Cytomegalovirus
CT	Computed Tomography
CTLA	Cytotoxic T-Lymphocyte antigen
DNA	Deoxyribonucleic acid
EMB	Endomyocardial Biopsy
FDG	Fluoro de-oxy glucose
FGF	Fibroblast growth factor
hNIS	human Sodium Iodide Symporter
HSV	Herpes Simplex Virus
^{123}I, ^{131}I, ^{124}I	Radioactive isotopes of Iodine
IFN	Interferon
IL	Interleukin
NK	Natural Killer (cells)
iNOS	Inducible Nitric oxide synthase
MBq	MegaBecquerel
MHC	Major Histocompatibility Complex

MRI	Magnetic Resonance Imaging
PDGF	Platelet-derived Growth Factor
PET	Positron Emission Tomography
RNA	Ribonucleic acid
SPECT	Single Photon Emission Computed Tomography
^{99m}Tc	Radioactive Technetium
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

SUMMARY OF THESIS

This thesis is based on a series of experiments aimed at designing a model of gene transfer to the transplanted heart. The use of viral vector-based gene therapy to target pathological processes following cardiac transplantation faces many challenges including the potential effects of the virus on the host as well as the need to establish the presence of the gene in the target organ. In the first set of experiments (Chapter 2), concerns over the effects of adenoviral gene transfer on the later development of cardiac allograft vasculopathy (CAV) were addressed. Heterotopically transplanted cardiac allografts from Brown Norway to Lewis rats revealed the presence of CAV at 120 days. *Ex vivo* adenoviral serotype 5 perfusion of the donor heart did not affect the later development of allograft vasculopathy.

In the second series of experiments, the feasibility of visualizing the presence of the sodium iodide symporter (hNIS) non-invasively following its gene transduction was established with the use of SPECT imaging. Following gene transfer, the recipients were injected with ^{99m}Tc in the first set of experiments (Chapter 3) or with radioactive ^{123}I (Chapter 4) and imaged under a SPECT scanner. Radioactive isotope uptake in the Ad-NIS group was significantly higher than in the group of animals whose hearts were perfused with just University of Wisconsin solution or with blank adenovirus without a marker gene. Sequential imaging of Ad-NIS-perfused hearts between post-operative days 2 and 14 revealed peak image intensity at day 5. Overall, image intensities correlated with *ex vivo* counts of radioactivity.

These data demonstrate that hNIS is an excellent reporter gene whose expression can be accurately and non-invasively monitored by serial radioisotope single photon emission computed tomography (SPECT) imaging.

1. INTRODUCTION

Section 1

1.1.1 Cardiac Transplantation and Complications

Heart failure remains one of the leading causes of death in the developed world. Heart failure affects about 900,000 people in the UK and this number is increasing due to improved prognosis of coronary artery disease and an ageing population. Even with optimal medical management, survival following discharge from hospital with a diagnosis of heart failure remains at 18 to 24 months[1]. Cardiac transplantation remains the only effective treatment for patients with heart failure refractory to conventional therapy.[2] About 10% of patients with heart failure are at an advanced stage and could potentially benefit from organ replacement.[3] Less commonly, heart transplantation is also recommended for recurrent life-threatening arrhythmias and angina refractory to other forms of therapy[2]. Over 3000 transplants were performed last year in over 300 countries. With an improvement in the understanding of the complications following this operation and better management, survival after transplantation has increased steadily with one year and ten-year survival rates close to 85% and 42.0% respectively[4]. Median survival among patients who survived the first year after transplantation was estimated at 13 years.[5] These outcomes following transplantation are more favorable when compared to other forms of therapy[6]. Despite the promising effects of transplantation, availability of organs remains a problem. At the end of June 2012, more than 3200 patients were on the waiting list in the USA with almost half of these patients waiting for more than 2 years.

More than 200 patients with heart failure died on the waiting list in the first half of this year alone. (www.unos.org) In the UK, there has been a significant decrease in the number of adult heart transplants performed in recent years[7]. Measures to increase the supply of donor organs for transplantation have not had the desired effect on the numbers of transplants performed each year in the UK[7].

Under these circumstances, it is imperative to ensure appropriate distribution of the available organs as well as optimum monitoring of the transplanted organs and quick and effective intervention in the case of any complications. The lack of sufficient donors to match the ever-growing need as well as the risk profile of the patients with failing transplants dictates the need to investigate and continuously refine alternative strategies to ensure long-term disease free survival.

While organ availability limits therapeutic options in those with end-stage heart failure, several potential complications could compromise the function of the graft after implantation. Primary graft failure accounts for upto 20% of the deaths in the first 90 days after transplantation[8, 9]. Infection and allograft rejection are other factors that could compromise graft function in the short term while malignancy and cardiac allograft vasculopathy (CAV) challenge long-term survival of the graft. Some of these complications are however amenable to therapeutic intervention that could prolong the life of the organ. With better donor management and therapy directed at expected complications, a 10-year survival of 42% has been achieved[4, 10]. There is however room for further improvement through implementation of strategies to prolong effective graft function.

The main obstacle to long–term survival of the allograft is cardiac allograft vasculopathy (CAV). This is characterized by concentric neointimal proliferation and ateriosclerosis leading to progressive narrowing of the lumen, myocardial infarction and graft dysfunction[11, 12]. More than 40% have some evidence of CAV by angiography at 5 years with CAV related adverse events (death and re-transplantation) accounting for 7% of patients post-transplant[13]. Therapeutic options are limited in this setting and no effective preventive strategy exists[14]. Attention has been focused on palliative measures to prevent clinical manifestations of CAV. Calcium channel blockers and Angiotensin converting enzyme (ACE) inhibitors have been shown to slow the initial development of CAV[15-18] due to their effect on hypertension, one of the risk factors for CAV[19]. Dyslipidemia, immunosuppressant drugs, oxidant stress and hypertension have an adverse effect on graft function[3]. An up-regulation of inflammatory mediators immediately following transplantation is also thought to contribute to the later development of allograft vasculopathy[20]. Coronary revascularization procedures including percutaneous transluminal coronary angioplasty (PTCA) [21, 22], coronary atherotomy and coronary artery bypass grafting (CABG)[12] Transmyocardial laser revascularization (TMR) have all been attempted to reduce morbidity due to ischaemia [23]. Retransplantation is an option although survival is shorter and the shortage of donor organs precludes this option in most instances.

Under such circumstances, gene therapy of the transplanted organ is a potential answer to the problems posed by pathological processes following transplantation. This concept of manipulation of gene profile of the patient in order to achieve a desired therapeutic effect is not new. Since the studies demonstrating the

feasibility of retroviral gene transfer into tumour infiltrating lymphocytes for the treatment of melanoma [24], gene therapy has come to be regarded as promising a cure not just for single gene mutations as originally conceived but a variety of acquired disorders [25, 26]. Effective gene transfer requires a vehicle of delivery of the therapeutic gene as well as a route of administration that ensures high transfection while not compromising normal organ function. The system used depends on the goal of genetic modification.

Gene Transduction is the abortive infection that introduces functional genetic information from the vectors into the target cell [27]. Gene transduction could therefore be construed as a system to supply cells with a gene product in a therapeutic quantity in order to alter the progression of disease. Under ideal circumstances, gene delivery would be specific to the region of interest with little promiscuous gene transduction therefore reducing the incidence of side effects.

1.1.2 Applications of Gene Therapy

Much of an enthusiasm to apply a gene-based approach to the treatment of cardiovascular disorders have been paralleled by dedicated efforts to understand the molecular basis for cardiovascular disease [25]. The vascular endothelium is effectively the largest organ in the body and is central to pathological processes affecting the cardiovascular system. Endothelium-targeted gene transfer strategies therefore have the potential of affecting diverse organ systems. Further discerning the physiology of the endothelium specific to a particular organ offers the possibility of modulating organ-specific pathology without the side effects of

systemic drug therapy. The problems associated with viral vector based gene therapy (outlined later) however remain. Consequences of an inflammatory response to the vector, incorporation of the DNA into the host cells, loss of gene expression and non-specific gene transfer are yet unsolved. However, gene transfer strategies are being investigated due to their potential to preserve endothelial function, prevent smooth muscle proliferation, maintain vascular tone, prevent a pathological vasospastic response, lower blood pressure in selected vascular beds, and stimulate therapeutic angiogenesis in peri-infarct regions after a myocardial infarction [28, 29]. An understanding of the molecular processes underlying development of collateral circulation in ischaemic myocardium and the availability of several growth factors like VEGF, PDGF, FGF, Ang-1 [30-34] that could create a local chemical milieu conducive to formation of collateral vessels and remodeling of the myocardium has led to an interest in therapeutic angiogenesis [29]. Angiogenic growth factors, cell cycle regulators and enzymes & receptors involved in lipoprotein metabolism have been transduced into the heart in an attempt to affect angiogenesis, thrombogenesis, in-stent restenosis, vascular graft occlusion and systemic and primary pulmonary hypertension [25, 28, 35]. Genetic modulation of intracellular calcium transport mechanisms [36], β-adrenergic receptor signaling and cardiomyocyte apoptosis are being targeted to restore and maintain ventricular contractile activity[37]. The enthusiasm of gene transfer for angiogenic growth factors [29] has however not translated to myocardial ischaemia in the context of CAV due to the diffuse nature of the disease. Evidence suggests that both humoral and cellular immune responses of the recipient to MHC antigens on the surface of the graft vascular endothelium lead to endothelial damage, a cytokine response and vascular smooth muscle cell

(VSMC) proliferation leading to neointimal hyperplasia.[38, 39] The pathological migration of VSMC from the tunica media to the intima with subsequent proliferation and concentric narrowing of the lumen leads to changes characteristic of allograft vasculopathy and has been the target of gene therapy attempts to modulate disease in animal models.

1.1.3 Gene Therapy of Transplanted Hearts

An understanding of the molecular mechanisms underlying cardiac allograft disease has therefore been followed by attempts to attenuate these processes and improve survival through gene transfer methods. [40, 41] Not surprisingly, the focus of gene therapy studies has been the abrogation of acute vascular rejection and improved graft survival. Murine cardiac allografts in IL-10 transgenic recipients demonstrated a reduction in graft-infiltrating CD-4 and CD-8 lymphocytes along with a reduction in intra-graft IL-2 and IFN-γ (attenuated Th1 response) leading to an absence of intimal lesions when compared to wild-type recipients. Lentiviral IL-10 gene transfer to cardiac allografts as well as Feline Immunodeficiency virus mediated viral IL-10 gene transfer combined with anti CD-3 monoclonal antibody have been shown to improve survival over a non-treated control group. [42] Adenoviral mediated transduction of a soluble Interleukin (IL-17) fusion protein (soluble IL-17R-Ig fusion protein) in donor hearts as well as the modulation of cellular immune responses through Ad-Haemoxygenase-1 gene transfer has been shown to delay acute allograft rejection and prolong the survival of cardiac grafts.[43] The protective effect afforded by such cytokine modulation seems to be associated with decreased numbers of macrophages, CD4+ T cells, and CD8+ T

cells infiltrating the graft along with reduced graft expression of IFN-g, TGF-b, IL-1b, and TNF-a. In addition, CTLA4Ig competitive inhibition of the T-cell co-stimulatory interaction of the T-cell CD28 with the APC molecules CD80 and CD86 has been attempted in an effort to attenuate acute vascular rejection in vascularised cardiac grafts. Experience gained with cytokine gene transfer in cancer studies [44] could thus be applied to transplantation models to create a graft-protective environment by the modulation of cellular and humoral allo-reactive pathways in graft rejection by delivery of immunosuppressive cytokines IL-10 and TGF-β. [45] Towards abrogation of CAV, adenoviral gene transfer of the dominant negative Rho-kinase has been shown to suppress neo-intimal formation, induce regression of constrictive remodeling and abolish the vasospastic response in coronary arteries. [46] In vivo gene transfer of oligonucleotides against Nuclear factor-KappaB (NF-κB), proliferating cell nuclear antigen (PCNA) and iNOS have also been encouraging in attenuating intimal hyperplastic lesions in animal models. [38, 47-49]

A shortage of donor organs has also spurred interest in the potential use of organs from other species. Xeno-transplantation could potentially provide an unlimited supply of donor organs although immune barriers limit its applicability at present. Expression of vascular adhesion molecules, chemokines and pro-inflammatory mediators in acute vascular rejection of xenografts [50] presents potential targets for vector-based genetic modulation in this setting.

1.1.4 Gene delivery to the Heart

One of the major determinants of the success of gene therapy trials is the proper selection of the appropriate vector to carry the gene with due attention to the time-course of the pathology being targeted. In addition, appropriate methods of gene transduction have to be studied in order to preserve function of the donor organ and prevent damage arising as a direct consequence of the gene transfer. An inability to translate the efficiency of gene transfer in cell cultures *in vitro* to organ systems *in vivo* has also prompted closer scrutiny of delivery techniques. [51-54]

Cardiac transplantation offers the opportunity to perfuse the heart with the gene after its removal from the donor and prior to its implantation into the recipient. The deleterious systemic effects of the viral vector on the donor are therefore avoided. Despite the potential advantages offered by *ex vivo* manipulation of the organ, conditions that are mandatory for organ preservation and for the reduction of ischaemic injury such as continuous cold perfusion and cold ischaemic storage do not always support efficient gene transduction. An obligation to maintain low perfusion temperatures to avoid ischaemic injury leads to inefficient gene transfection. Evidence suggests inefficient endothelial and vascular smooth muscle transduction under hypothermic conditions compared to 37˚C, where nearly 100% cardiomyocyte transduction is feasible under appropriate perfusion conditions. [55, 56]

Perfusion of the donor organ with the gene-carrying vector is followed by its transfer across the vascular endothelium. Efforts at more efficient cardiomyocyte transduction have included pre-treatment with serotonin and bradykinin prior to *ex vivo* perfusion with Ad-βGal [57] towards modulation of endothelial permeability. Use of a calcium-poor perfusate has also been reported to result in a greater efficiency of gene transfer, as the integrity of the endothelial tight junctions is calcium-dependent. Previous studies have reported an improvement in gene transfer efficacy from 5% to 67% with the pre-treatment of the heart with calcium-free solutions. Use of such pharmacological manipulation to increase endothelial permeability facilitates the passage of macromolecules and virions into the myocardium. [58] Although such attempts have been made to develop a model for gene transfer during cardiac catheterization, this concept could be used to ensure efficient transduction with an *ex vivo* perfusion technique. [57, 59]

Although direct injection of the viral vector into the myocardium offers an easily replicable mode of delivery that is not dependent on trans-endothelial transfer and results in efficient gene transduction, [60-63] gene expression is confined to the needle track. [63] Uniform gene expression is ensured with the use of intra coronary infusion of the virus either as a bolus dose [64] or with the use of a perfusion apparatus [56, 65]. Cardiac transplantation presents a unique situation in that the organ of interest is available for *ex vivo* genetic manipulation prior to re-implantation in to the recipient. Pelligrini et al. have demonstrated efficient gene transfer using an *ex vivo* perfusion technique in rat hearts. The feasibility of this technique has been duplicated in a large animal model with efficient gene transduction occurring with a concentration of 10^9 pfu/ml of Adenovirus in the

perfusate. The side effects resulting from promiscuous infection of other organs like the liver and spleen with adenoviral vectors are avoided with this technique. More efficient gene transfer has been demonstrated with a hypothermic ex-vivo perfusion technique compared to a single bolus dose of the vector [45, 65]. Studies have reported better recovery from ischaemia-reperfusion injury in rat hearts following transduction with Adenoviral mediated Manganese superoxide dismutase (Mn-SOD) using a continuous hypothermic reperfusion system as compared to a high pressure infusion of the same virus down the coronaries through the aortic root. [66]

Limitations of available delivery techniques include inefficient transfection of endothelial cells and expression of the gene predominantly in sub-epicardial regions of the heart reflecting the propensity of this region to rewarming [55]. The use of *ex vivo* coronary perfusion of the heart at 4˚C [45] reported an uneven distribution of the transgene, with a preference towards sub-epicardial perivascular regions. Intermittent external compression of the left ventricle during perfusion resulted in a more homogenous distribution of gene transduction throughout the myocardium reflecting the dependence of this technique on an optimum perfusion pressure. In larger animal gene transfer experiments where perfusion pressures are more reliably monitored, reports suggest an interesting phenomenon where the flow rate had to be increased in increments in order to maintain a constant perfusion pressure due to progressive coronary vasodilatation. It has not been determined whether perfusion rate or pressure dictates the efficiency of gene transfer. A higher efficiency of reporter gene transfection with higher flow rates has been hypothesized to being due to opening of pre-capillary

sphincters at a critical perfusion pressure [56]. In both the native and transplanted hearts, catheter-based delivery of the gene is an attractive concept although the presence of blood, single-pass through the coronary arteries, spillover into the systemic circulation and the inability to alter perfusion pressures would be distinct disadvantages. The transient nature of gene expression and the absence of a therapeutic effect despite efficient gene transduction [67] remain valid concerns. In addition, evidence of differential gene expression in diseased arteries versus those with an intact endothelium [68] and therapeutic effects of gene transfer with just adventitial rather than endothelial transduction [69, 70] have highlighted a greater need for *in vivo* models of disease to investigate the effects of therapeutic genes.

Another problem associated with the interpretation of *in vitro* data includes distinct properties of cells in culture compared to conditions *in vivo*. 'Normal' cells in culture may also not be accurate representations of an *in vivo* disease model. The effects of anatomical barriers like the internal elastic lamina, receptor expression in disease and mechanism underlying poor gene expression despite efficient infection of cells are better understood through experiments carried out *in vivo*.

In addition to the above, the biological effects of gene transfer are dependent upon the delivery technique. Differential changes in cytokine profiles, APC-T cell interaction and cellular immune responses and local graft inflammation have been noted with different routes of administration. For instance, intravenous Haemoxygenase-1 gene transfer into rats bearing heterotopic cardiac allografts

resulted in decreased TNFα, IFNγ, IL-10 and iNOS transcripts while intra-graft intramuscular injection did not alter the graft cytokine mRNA profile in the same manner. All available techniques for cardiac gene delivery have drawbacks associated with uniform gene expression, high transfection efficacy or ease of clinical translation. In addition, cell-type specific gene expression must be achieved in order to affect cellular processes. Engineering the system to target the molecular process in question is therefore important in order to be able to track the benefits as well as to monitor and avoid side effects of the therapy. While most pathological processes in the graft are orchestered around the vascular endothelium, vasomotor dysfunction following adenoviral transduction of the endothelium is an issue that has been closely followed in pre-clinical trials. [71, 72]

1.1.5 Immune Responses to Vectors and their Implications

Alongside the search for the perfect delivery technique, investigators have concentrated their efforts on finding a carrier molecule that delivers the gene reliably, consistently and efficiently to the target organ while remaining biologically inert. Although such a molecule does not exist, progress in achieving desirable levels of gene expression has been made with the use of viral vectors. However, the biggest obstacle to virus-based gene transfer is the potent immune response elicited by the vector as well as the transgene. [73-77] This is exemplified in studies involving serotypes of adenoviruses.

Adenoviridiae are non-enveloped viruses with a 30-40kb double stranded DNA fragment and have been natural choices for DNA bearing vectors due to their

ability to transduce differentiated non-dividing cells, ability to be generated in large titres and resistance to complement mediated inactivation *in vivo* [78]. First generation adenoviruses were engineered for gene therapy application by deletion of their E1 region to render them replication-defective as well as by deletion of E3 to create room for addition of therapeutic genes. Second generation vectors are further devoid of E2 and E4 regions to enable addition of more gene fragments and blunt the innate immune response to viral antigens. Despite this, the body's immune response to the vector and the gene product leads to an unstable and short-lived transgene gene expression in living cells. [77, 79-82]

Entry of adenoviral vectors into the recipient is followed by the induction of a non-specific innate immune response largely independent of transgene expression. The first line of defence against foreign antigens is the rapid scavenging of viral particles by NK cells, macrophages and dendritic cells [83]. The activation of these cells leads to release of inflammatory cytokines like IL-6, IL-12 and TNF-α independent of viral or transgene expression. [84, 85] In most adenoviral serotypes, this response is triggered by signaling pathways set in motion upon viral entry into cells following integrin-RGD binding. The dominant receptor promoting cellular invasion is the coxackievirus adenovirus receptor (CAR), a peptide expressed on the cell membrane.

Antigen specific responses to both viral proteins and the transgene limit the extent and duration of gene expression in target cells. Low levels of viral gene expression leads to MHC-1 restricted activation of cytotoxic T lymphocytes and a rapid elimination of transgene expression. The antigen presenting cells (APC)

also stimulate the secretion of cytokines like IL-6 and IL-12 that, in addition to evoking a rapid local inflammatory response also activate cytotoxic T-lymphocytes against the infected cells. Depletion of APCs attenuates both the innate immune response as well as the cytotoxic lymphocyte responses indicating the intersection of these pathways [86]. The development of an immune response could depend on the antigen presenting ability of the organ and the low dendritic cell content of the heart may blunt the immune response against the transplanted organ. The obligate use of anti-T cell immunosuppressant drugs in transplantation could also ensure a more prolonged transgene expression in the myocardium [87]. However, immune responses to viral structural proteins as well as to the transgene product limit gene expression in the transplanted heart.

Concerns over the effects of the adenovirus serotype 5 vector on the transplanted heart, in particular the later development of CAV following an initial immune response were addressed in the study described in chapter 2.

Section 2. REPORTER GENES AND MOLECULAR IMAGING

1.2.1 Reporter Genes

The challenges to gene therapy can be monitored and overcome by a careful selection of vector systems that are targeted to the tissue as well as tailored to the myocardial pathology. Temporal association of gene expression with the course of the pathology and appropriate analyses of functional effects of the gene can only be attempted in the presence of reliable reporter systems that reflect the presence of the gene. The commonly used 'Lac Z' reporter gene allows visualization of the β-Gal gene product by histochemical analysis but this concept has had some drawbacks. Histochemical analysis of the function of the enzyme as opposed to the presence of the gene by anti-body labeling has been shown to underestimate gene transduction both *in vivo* and in stably transfected cells in culture[88]. In addition, it has been shown in the rabbit myocardium that areas of micro-infarction stained positive for X-Gal following cationic liposome mediated gene transfer despite the absence of the gene in those areas[89]. To date the commonly used reporter genes in preclinical studies have required sacrificing the animal to obtain tissue sections for *in vitro* analyses of the organ including mapping of the reporter gene. Investigators have therefore attempted the use of reporter genes that generate an optical signal that could be captured without sacrificing the animal[90, 91]. Such fluorescent and bioluminescent reporters (e.g. firefly luciferase, green fluorescent protein) are specific to the tissue expressing them and have limited background noise. However, emission wavelengths between 450-500nm afford limited tissue penetrability of the order of a few millimetres. Reasonable visualization in rodent models can therefore not be

translated to large animal gene transfer experiments[92]. In addition, low spatial resolution implies absence of any reliable anatomical correlation of the optical signal. These drawbacks could be overcome with the use of magnetic resonance imaging, which offers excellent spatial resolution and anatomic detail with the absence of exposure to ionizing radiation. However, a low specificity for the contrast material and long image acquisition times are distinct shortcomings that need to be addressed.

Questions have also been raised over the closeness with which animal models of disease correlate with human conditions. Anatomical heterogeneity of the atherosclerotic lesion as well as the complexity of vascular responses to endothelial injury in the form of VSMC proliferation, neo-intimal hyperplasia, apoptosis, matrix remodeling make it difficult to produce animal models that simulate human disease conditions[93]. An apprehension in translating principles demonstrated in rodent studies to large animal models has also resulted in a paucity of such data resulting in an uncertainty in the direction of gene therapy in cardiac transplantation.

Given the limitations of currently available gene transfer strategies as described, it is important to be able to measure the efficiency of gene transfer and track gene expression over time in order to correlate it with functional studies that monitor the presence of a desired therapeutic response. These studies are better carried out in large-animal models of disease. The development of imaging strategies like Positron Emission Tomography (PET) and SPECT makes it possible to track molecular processes and pathology in a non-invasive fashion in these models. These models could be used to assess gene transduction efficacies and

effectiveness of gene delivery techniques in real-time[94, 95]. This technology would be especially useful in diseases where long-term gene expression is warranted and fading of gene expression could be followed up by a second dose of a therapeutic gene.

1.2.2 Molecular Imaging

The increasing use of imaging modalities like Duplex Ultrasonography, CT angiography, MR angiography and arteriography to diagnose cardiovascular pathology as well as adjuncts for invasive therapy[96] has presented the possibility of using this expertise in gene therapy trials. Molecular imaging of cardiovascular processes assumes the presence of a molecular target to which can bind a suitable antibody or peptide ligand. This binding then generates a signal that can be captured non-invasively by an appropriate imaging modality, the choice of which depends on the ligand and on the depth and spatial resolution that is necessary.

Use of Magnetic resonance imaging with gadolinium[97] and ultrasonographic monitoring of microspheres [98] has permitted real-time imaging of the catheter-based gene delivery process, to ensure successful delivery of genes and the absence of complications associated with the procedure. Echogenic microspheres could be visualized with relative ease using ultrasonography and have the added potential of doubling as a non-viral vector. Ease of translation of an already widely used imaging modalities like ultrasound and computed tomography to gene therapy applications makes it an attractive prospect. These imaging techniques also have the ability to provide higher spatial resolution and therefore

greater anatomical correlation with molecular processes. Investigators have used MRI to track *in vivo* processes like apoptosis and to characterize vulnerable atherosclerotic plaques by capturing superparamagnetic iron oxide (SPIO) nanoparticles [99, 100].

An interest in non-invasive imaging of myocardial perfusion and viability has played a major role in the development of nuclear imaging modalities in cardiovascular medicine [101-103]. In PET scanning, an array of circular detectors captures positron emission from a radionuclide in a defined 3-dimensional space. Tomographic reconstruction of this emission enables imaging of the organ or region of interest. In cardiac imaging, there is a wide choice of radiopharmaceuticals (oxygen-15, nitrogen-13, carbon-11, fluorine-18) that can be tagged to fuel substrates, hormones or receptors to enable imaging of functional processes of interest. Non-invasive imaging of gene expression could be superimposed on perfusion imaging of the myocardium obtained at the same time [60, 104] to determine therapeutic efficacy of gene transfer in the myocardium.

However, the drawbacks of PET imaging include the availability of PET imaging equipment and a short half-life of radiotracers. This necessitates the on-site presence of expensive cyclotrons to generate the radioisotopes. Satellite PET centers are being developed to circumvent this problem. In addition, the substrate to which the radioisotope is labeled should be carefully chosen based on the functional process under study. For example, glucose tagged to fluorine-18 has been used in the past to study myocardial perfusion. However, its low uptake and rapid clearance due to participation in multiple metabolic pathways has led to the

search for a particle that is trapped within the myocardium long enough to permit satisfactory imaging. Although it promises to enhance the sensitivity of imaging of processes the drawback of PET imaging is the current inability to produce images of high resolution that are possible with more conventional forms of imaging. This has origins in the development of PET as a modality to image molecular function rather than to visualize clear anatomical landmarks. In oncology, where PET applications have the most potential, the inability to visualize clear landmarks poses a definite problem. The use of dual-modality imaging with PET/CT has attempted to address this issue [105]. Clinical studies exploring applications of PET and PET/CT have been encouraging[106, 107].

In clinical cardiac transplantation, the invasive nature of endomyocardial biopsies (EMB) to monitor rejection has encouraged the investigation of non-invasive imaging modalities in diagnoses. In acute rejection, the possibility of a lag-time between the presence of alloreactive lymphocytes and myocardial damage detected by EMB has been suggested as a potential advantage. Somatostatin receptors expressed by activated lymphocytes were visualized by the somatostatin analogue ^{111}Indium pentetreotide at least 1 week prior to histological evidence of rejection by EMB [108]. Detection of myocyte apoptosis by ^{99m}Tc-labelled Annexin V and radiolabelled anti-myosin antibodies have been proposed as an alternative to EMB[109-111]. Although these techniques are feasible and sensitive to the interrogated pathology, overall value in diagnosing rejection and dictating treatment remain undetermined. The advantages of non-invasive imaging of molecular processes have also been extended to the visualization of chronic rejection. Monocyte chemo attractant protein-1 (MCP-1) is a potent chemokine

secreted by graft infiltrating monocytes and activated vascular endothelium. MCP-1 is associated with vein graft intimal hyperplasia[112] and binds to its receptor CCR-2 in the context of graft atherosclerosis and pro-inflamatory conditions. In a rodent model of CAV, ^{99m}Tc visualization of these receptors found them to be up-regulated in grafts with evidence of vasculopathy [113]. There is evidence to suggest a role for myocardial perfusion imaging with Tc-sestamibi SPECT in conjunction with resting echocardiography to identify early graft coronary artery disease in the transplanted heart [114]. Another application of nuclear imaging has been the assessment of sympathetic renervation following transplantation. Denervation of the heart during procurement results in a loss of exercise performance, ventricular function and sensation of chest pain to reflect coronary artery disease following transplantation. Sympathetic renervation of the ventricles is heterogenous and occurs at a variable rate following transplantation. Nuclear imaging of myocardial perfusion using radiolabelled NE analogues like MIBG and ^{11}C-Hydroxyephedrine has been used to follow this process in a non-invasive manner [115, 116].

Conventional models to study gene transfer have had several limitations some of which have been described in detail earlier. The absence of a non-invasive imaging modality to trace and track gene transfer has led to interest in nuclear imaging of gene expression. By this approach, it is possible to perform longitudinal analyses to answer questions related to the site and duration of gene expression as well as safety concerns arising from promiscuous gene transduction without sacrificing the animal. In addition, this approach allows for the more authentic *in vivo* tracking of organ perfusion, drug kinetics as well as molecular

processes like cell death and angiogenesis [117, 118]. Non-invasive tracking of reporter genes could be accomplished by one of two broad approaches available currently. The "direct" approach uses radioligands that are concentrated by the therapeutic gene product. In the "indirect approach", a reporter gene is tagged to the therapeutic gene to perform the same function. This second approach allows greater flexibility in choosing the reporter gene and obviates the need to design a probe/gene imaging system for every therapeutic gene of interest. The gene product could be an intracellular enzyme, a membrane transporter a cell surface antigen or receptor or a fluorescent protein. HSV1-tk and HSV-sr39tk gene products phosphorylate compounds including acycloguanosines (e.g., acyclovir; ganciclovir, GCV; 9-[4-fluoro-3- (hydoxymethyl) butyl]guanine, FHBG) [104, 119] and thymidine analogues (e.g., 5-iodo-2′-fluoro-2′deoxy-1-β-D-arabino-furanosyl-uracil, FIAU) [60]. Radiolabelled compounds can be visualized with PET scanning to track gene-transfected cells [60, 120]. The use of 3-(2′-[^{18}F] fluoroethyl)spiperone reporter probe with the human Dopamine 2 receptor gene (hD2R) [121, 122] and ^{111}In-DTPA-octreotide with the human somatostatin receptor subtype-2 (hSSTR2) gene [123] are other examples of non-invasive reporter systems.

1.2.3 Radioprobes

The sensitivity of *in vivo* signal intensity in predicting the level of gene expression depends on the probe used in the study. The presence of a response relating radioligand uptake to the density of receptors or mechanism of concentration of the probe by the tissue is important. Specificity of uptake should also be addressed in preclinical studies by either pharmacological blockade or the

use of appropriately designed negative controls. The imaging modality of choice should reflect the pathology under study as well as the technology available at a particular site. The imaging process as well as the radiotracer should interfere as little as possible with the progression of the pathology as well as the metabolism of the organ under study. In addition, it is necessary to determine the kinetics of the radiotracer within target organs as tissue-specific expression of enzymes could affect intracellular tracer accumulation.

The feasibility of non-invasive imaging of therapeutic IL-10 gene transfer was demonstrated using HSV1-sr39tk reporter gene with 9-(4-[^{18}F] fluoro-3-hydroxymethylbutyl) guanine ([^{18}F] FHBG) visualized with PET myocardial imaging. [124] The disadvantages of HSV-tk to image gene transfer however include an immune response developed by the host due to the recognition of the gene product as non-self. Also, with the enzyme being intracellular, transport of the radioisotope through the cell membrane could be the rate-limiting step.[125, 126]The human norepinephrine transporter (hNET) has recently been investigated as a reporter gene with the ^{11}C-m-hydroxyephedrine (^{11}C-mHED) probe imaged with PET in a rat tumour model. hNET is a membrane transporter that facilitates reuptake of norepinephrine at the synapse. ^{11}C-mHED uptake in the hNET transfected tumors correlated closely with tumour expression of hNET and was specific for hNET [127]. This system is however inapplicable to cardiac gene transfer due to the inherent presence of the transporter in the normal heart. In transplanted hearts, the variable rate of sympathetic renervation and NET expression implies the absence of reliable negative controls and limits the clinical applicability of such a technique.

1.2.4 Role of NIS in Non-invasive Molecular Imaging

The thyroid gland is an endocrine organ producing two active iodine-containing hormones, Tri-iodothyronine (T_3) and tetra-iodotyronine (T_4, Thyroxine). These hormones have a wide spectrum of actions including proper development of the central nervous system in neonates. An efficient system of concentrating iodide is an essential initial step in the synthesis prior to organification and incorporation of iodine into thyroglobulin molecules to form thyroxine. This has long known to be due to the expression of the Sodium Iodide Symporter (NIS) on the baso-lateral membrane of the thyroid follicular cells. The Symporter works to concentrate iodide from circulating blood by using the energy generated by transporting sodium ions down their concentration gradient into the cell. One iodide ion is transported for every two sodium ions. A sodium gradient exists naturally due to the activity of the Na^+/K^+ ATPase that pumps 3 Na^+ ions out of the cell for every 2 K^+ ions moved into the cell. This is an energy dependent pump, the integrity of which is vital to normal activity of the NIS [128]. It has been estimated that an iodide gradient of 20-40 exists between the plasma and thyroid follicular cytoplasm due to the expression of NIS. Other anions like technetium are also transported by the NIS into the cell. This inherent anion-concentrating ability has been utilized in nuclear imaging of the thyroid gland following injection of radioactive tracers like ^{123}I and ^{99}Tc. Radioiodine concentrating-ability of NIS has been harnessed to ablate malignant thyroid tissue following thyroidectomy, to destroy follicular cells in patients with toxic multinodular goiter and to perform whole-body scans to locate thyroid tissue [129]. The isolation

and cloning of the rat NIS gene from the Fisher rat thyroid cell line (FRTL-5) in 1996 [130] opened up the possibility of using the NIS gene in non-invasive imaging of gene transduction. In addition, NIS gene transfer was used to concentrate radioiodine and selectively destroy murine liver and colon cancer and human melanoma cell lines. Scholz et al. [131] demonstrated stable transfection of HCT 116, a human colon cancer line with Ad-NIS driven by the tumour-specific CEA promoter. Subsequent exposure to I^{131} killed approximately 95.6% of NIS-expressing cells demonstrating the potential of NIS as a therapeutic gene in radioiodine treatment of colon carcinoma. Enhanced radioiodine uptake was also demonstrated in hNIS expressing prostate adenocarcinomas implanted subcutaneously in rats [132]. Other studies have demonstrated a similar enhanced radioisotope uptake in breast carcinoma [133, 134], head and neck squamous carcinoma, prostate carcinoma [135, 136], colorectal adenocarcinoma, [136], ovarian cancer [137] multiple myeloma [138] and medullary and dedifferentiated thyroid cancers [139] following NIS gene transfer. Imaging of pulmonary gene transfer with the use of PET with ^{124}I and γ-camera has been demonstrated. [140] Other advantages of the use of NIS as a gene transfer reporter include the absence of an immune response to the gene product due to the presence of NIS in the human thyroid and the expression of the symporter on the cell surface facilitating direct contact with the probe in circulating blood.

NIS could therefore be used as a non-invasive reporter gene in cardiac transplantation.

1.2.5 Thesis Aims

The hypothesis under investigation was that non-invasive modalities could be used in the monitoring of gene transduction in cardiac transplantation.

The specific aims were

1. To address concerns over the effects of the adenovirus serotype 5 vector on the transplanted heart, in particular the later development of CAV following an initial immune response

2. To demonstrate the feasibility of using SPECT to image gene transduction following Ad-NIS gene transfer in a model of cardiac transplantation

Chapter 2

THE EFFECTS OF ADENOVIRAL GENE TRANSFER ON CARDIAC ALLOGRAFT VASCULOPATHY

ABSTRACT

Introduction

Adenovirus serotype 5 has remained the preeminent vector in pre-clinical gene therapy applications in cardiac transplantation. Concerns over the potential effects of adenoviral vectors on the later development of cardiac allograft vasculopathy (CAV) were addressed in this study.

Methods

Hearts (n=22) harvested from Brown Norway rats were perfused *ex vivo* with either UW solution with no virus, Ad-CMV-LacZ or Ad-CMV-Null. Donor hearts were transplanted heterotopically into the abdomen of Lewis rats. All recipients received Cyclosporine for the duration of the experiment. Transplanted hearts were recovered for analyses at 120 days. Sections of the heart were stained with elastic Van Gieson stain for morphometric analysis of the vessels to ascertain the degree of vascular luminal occlusion. Haematoxylin-eosin staining facilitated diagnosis of chronic rejection.

Results

77% of transplanted hearts showed signs of chronic rejection with no difference in the proportion of animals between the groups (p=0.797). No difference was noted in the degrees of vascular luminal occlusion between Ad-Null (0.57±0.22), Ad-LacZ (0.62±0.19) and UW groups (0.47±0.29) (p=0.653).

Conclusions

Vascularized cardiac allografts transplanted from Brown Norway to Lewis rats demonstrate the presence of CAV at 120 days. Adenoviral perfusion of the donor heart *ex vivo* does not affect the development of CAV.

Key words: Gene therapy, Adenovirus, Cardiac allograft vasculopathy, Heart transplantation

2.1 INTRODUCTION

With an estimated incidence of 50% at 5 years, cardiac allograft vasculopathy (CAV) is the biggest impediment to long-term disease free survival after cardiac transplantation.[141] Limited success with conventional therapy has led investigators to consider viral vector-directed gene therapy approaches to reduce the incidence and progression of this disease.

However, viruses have been implicated in the development of CAV, chiefly through their effects on graft endothelial function.[142] The expression of adenoviral proteins in pediatric allografts demonstrating the presence of transplant vasculopathy [143] and the adverse effects noted with adenoviral infections in immunosuppressed patients[144] have led to concerns over the use of adenoviral vectors in pre-clinical gene therapy applications in cardiac transplantation.[145] Although Adenovirus serotype 5-based transduction of genes have shown promise in affecting transplant vasculopathy [146-149] and prolonging cardiac allograft survival,[150, 151] adenoviral gene transfer is known to elicit a potent innate and acquired humoral and cellular inflammatory response[84, 152-154] that could trigger changes leading to CAV. Therefore, the effect of the virus on cardiac vasculature needs to be ascertained prior to assessment of the effects of therapeutic genes on graft vasculopathy.

In this study, we attempted to address concerns over the effects of the viral vector on the later development of CAV following *ex vivo* perfusion of the donor heart.

2.2 MATERIALS AND METHODS

Animals

Inbred Lewis rats (250-300g, $RT1^{l}$) and Brown-Norway rats (250-300g, $RT1^{n}$) were used as recipients and donors, respectively, for allogenic abdominal heterotopic heart transplantation. Procedure and handling of animals were reviewed and approved by the Institutional Animal Care and Use Committee of Mayo Clinic and Foundation in compliance with "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and Published by the National Institute of Health (National Institute of Health publication No.86-23, revised 1985).

Adenoviral vectors

A replication defective E1a-deleted serotype 5 adenoviruses encoding for non-nuclear targeted Escherichia Coli β-galactosidase under the control of the CMV promoter were used in this study. Ad-Null, an identical vector not containing the β-Galactosidase expression cassette was used to ascertain the effects of the vector independent of the transgene. (Ad-CMV-LacZ and Ad-Null, provided by James Wilson, Institute for gene therapy, University of Pennsylvania).

Experimental Groups

Rats were randomly assigned to one of three experimental groups. Hearts were harvested and perfused *ex vivo* using techniques described previously.[65] Briefly, cardioplegia was obtained by infusion of 5 ml of UW solution through the cannula inserted in the innominate artery, the heart was harvested and the pulmonary artery was cannulated with a 14G cannula. With the inflow cannula in the innominate artery and the outflow cannula in the pulmonary artery, 5 ml of UW were recirculated at 4°C for 30 minutes. In group C (control group) (8 animals) hearts were perfused with UW solution as previously described [65] at a flow of 0.75 ml/min. In group A (Ad-Null group n=6) and B (β-Gal group n=8 animals) hearts were treated as in group C with the difference that in UW were diluted $3.5x10^8$ pfu (total) of Ad-Null and Ad-LacZ, respectively. After 30 minutes of perfusion, hearts were stored in UW solution at 4°C during recipient preparation.

Recipient operation

Preparation of the recipient and heterotopic abdominal heart transplantation were performed as previously described.[155] All rats received post-operative analgesia. All animals received 5 mg/Kg of cyclosporine A 3 times a week intramuscularly for the duration of the experiment.

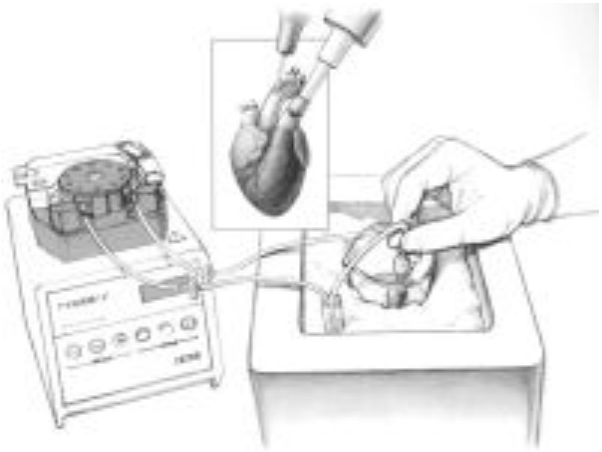


Illustration of the gene delivery system used in the current study (above)

The solution containing the adenoviral vector was perfused from a vial through the vasculature of the donor heart and back to the vial by means of a peristaltic pump *(left)*. The container with the heart immersed in UW solution and the vial with the viral solution were kept on ice during the circulation time. The *inset* depicts the inflow and outflow catheters placed in the aorta and pulmonary artery respectively.

Presence of graft coronary disease

After 120 days all rats were sacrificed by deep pentobarbital anesthesia (70 mg/kg) the transplanted hearts were harvested. Mid ventricular sections were fixed in formalin and routinely processed for histopathological analysis. Sections were cut and placed in OCT compound, snap frozen in liquid nitrogen and stored for immunohistochemical analyses.

Two routinely processed sections for each animal were stained with haematoxylin and eosin (H&E) in order to determine the presence of chronic rejection. In order to assess the degree of vessel occlusion slides were stained with elastic Van Gieson to highlight the elastic lamina (EL). Five slides were prepared for each rat, and the occlusion ratio of each vessel able to be visualized was recorded. The degree of intimal hyperplasia was morphometrically assessed by using the formula: luminal occlusion= (Internal elastic laminal area-luminal area)/Internal elastic laminal area.[156] Over 1000 vessels were analyzed for neointimal proliferation with an average of 50 vessels from each animal. Vessels of hearts with no histological evidences of chronic rejection were considered negative controls.

Statistical Analysis

The mean occlusion ratios for each rat were compared using analysis of variance (GLM procedure) to look for an overall difference between the groups. A mixed effects model was used to make comparisons between pairs of groups using every measurement for every rat, treating individual rats as random effects. The proportion of animals with evidence of rejection in each treatment group was compared using Fisher's exact test. All data are expressed as mean ± SD. A p value of <0.05 was consider significant.

2.3 RESULTS

All animals completed the protocol.

17 of 22 (77%) transplanted hearts showed evidence of chronic rejection on H&E stained slides. No difference was noted between the groups in the proportion of animals showing evidence of chronic rejection (UW 3/8, Ad-Null 1/6 and Ad-LacZ 1/8, p=0.797). The average number of vessels visualized per rat were 49.4 ± 13.2 in the UW group, 39.2 ± 10.3 in the Ad-Null group and 60.1 ± 8.1 in the Ad-LacZ group. Specimens from rats in UW-treated group had fewer measurable vessels than from rats in other groups (p=0.017) Mean occlusion ratios in the groups were as follows: Ad-Null (0.57±0.22), Ad-LacZ (0.62±0.19) and UW groups (0.47±0.29) Comparison of the means occlusion ratios between the groups did not reveal any significant difference (p=0.511). (Table 1 and Figure 1)

2.4 DISCUSSION

These results confirm that vascularized cardiac grafts from Brown Norway to immunosuppressed Lewis rats demonstrate the presence of CAV at 4 months. *Ex vivo* Perfusion of donor hearts with either the adenovirus serotype 5 expressing LacZ or Ad-Null at the time of transplant did not affect the development of graft vasculopathy. Studies have reported the development of neo-intimal proliferation in both abdominal aortic allografts and vascularized cardiac grafts[157] from Brown Norway to Lewis rats. The development of CAV in the face of anti-T cell therapy in this study simulates the clinical situation more closely.[158]

The pathophysiology of cardiac allograft vasculopathy has yet to be fully understood. Leading hypotheses suggest that incompletely suppressed immune and non-immune factors initiate cytokine and growth factors cascades that evoke endothelial injury leading to neo-intimal hyperplasia and VSMC proliferation.[12, 40, 159] Viral vectors, by up-regulating endothelial adhesion molecules and promoting T-cell infiltration at the sites of gene transfer could provoke endothelial injury and stimulate graft vasculopathy. This effect was not confirmed in our comparisons of hearts perfused with Ad-Null with those perfused with UW solution. However, Newman et al[153] have reported a positive correlation of adenoviral transduction with the development of intimal hyperplasia in rabbit femoral arteries. This could be due to our using a lower dose of the vector and perfusing the heart under hypothermic conditions *ex vivo*. Studies using *ex vivo* perfusion of the heart under hypothermic conditions have revealed an absent[87] or mild[64] inflammatory response. Chan et al[160] have also suggested that an

inflammatory response following adenoviral transfection is organ specific and that the low antigen-presenting cell (APC) content of the heart might favour long-term gene expression. It is possible that suppression of inflammation due to adenoviral infection with Cyclosporine prevents the acceleration of CAV. It is also likely that the degree of neo-intimal proliferation is dependent on the model. Although the profile or distribution of transfected cells in the heart or the efficiency of gene transduction have not been defined in this study, our previous experience with this perfusion technique has demonstrated a gene transduction efficacy of 1.8% in the sub-endocardial region to 45 % in the sub-epicardial region, with the transgene showing a definite preference to the cardiomyocyte over the vascular endothelium.[64,87]

The sections of myocardium were not separately analyzed to confirm transduction as this model of hypothermic *ex vivo* perfusion has been validated by previous experiments conducted in our lab.[64,65] Sections of the myocardium examined for transduced gene revealed gene expression accentuated in myoctes in the sub-epicardial region and the right ventricular wall. Perfusion of the heart for 30 minutes through a pump enables more efficient gene transduction than that following a bolus injection of the virus. In addition, examination of the samples for ischaemic damage revealed greatest injury to the myocardium following high-pressure bolus injection. Continuous low-pressure perfusion ensured better myocardial protection against ischemic injury. This technique was therefore adapted for this study.

Statistical analysis was performed pair-wise between groups, under a 'random-effects' model, using all the sections taking the mean occlusion ratios from each perfusion group. Limitations of this study include the presence of fewer vessels in

the sections from UW-perfused hearts compared to hearts from the other groups. The potential criticism of this study not being adequately powered to note a difference between the groups, i.e the possibility of a type II error has been noted. However the trend towards lower occlusion ratios from the vessels from the UW-perfused hearts was not significant.

More recent studies[161] have reported functional effects of adenoviral gene transfer using a similar hypothermic *ex vivo* perfusion technique emphasizing the importance of assessing qualitative rather than quantitative end points of gene transfer. Although short-term expression of adenoviral vector based genes limit their use to attenuation of processes like ischaemia-reperfusion injury or acute rejection, this study did not identify a justification for concerns over the effects of the immune response elicited by the vector on the later development of graft vasculopathy.

Conclusions

Heterotopically transplanted cardiac allografts from Brown Norway to Lewis rats reveal the presence of CAV at 120 days. This study did not identify a correlation between perfusion of the donor heart with *ex vivo* adenoviral serotype 5 and the later development of allograft vasculopathy.

2.5 TABLES AND FIGURES

Treatment Group	Number of animals	Average of the mean ratios ± SD
A. Ad-Null	6	0.57 ± .22
B. Ad-LacZ	8	0.62 ± .19
C. UW	8	0.47 ± .29

Table 1

Distribution of occlusion ratio for individual rats across the groups

Comparison of the means occlusion ratios between the Ad-Null (0.57±0.22), Ad-LacZ (0.62±0.19) and UW groups (0.47±0.29) groups did not reveal a significant difference (p=0.511) (mean±SD, Analysis of variance)

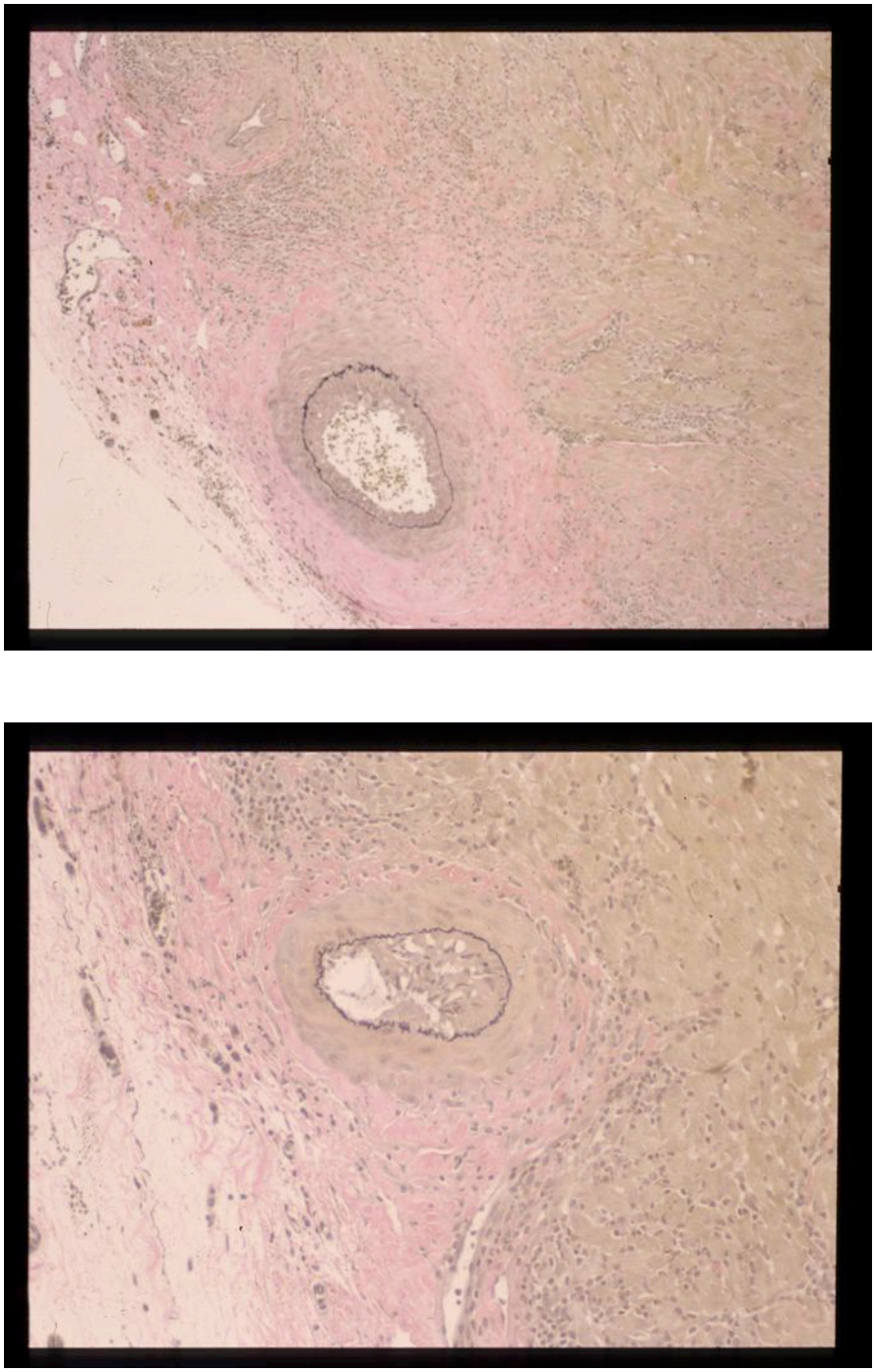


Figure 1. Images of coronary arteries demonstrating the presence of CAV in both Ad-LacZ (top) and UW-perfused (bottom) hearts Morphometric analysis did not show significant differences in occlusion ratios between Ad-Null (0.57±0.22), AdLacZ (0.62±0.19) and UW groups (0.47±0.29) (Data expressed as Mean ±SD)

Chapter 3

NUCLEAR IMAGING OF GENE TRANSDUCTION IN THE TRANSPLANTED HEART WITH SODIUM IODIDE SYMPORTER (hNIS) and ^{99m}Tc

Nuclear Imaging of Gene Transduction in the Transplanted Heart with Sodium Iodide Symporter (hNIS) and ^{99m}Tc

ABSTRACT

Introduction

NIS permits concentration of iodide within the cell on which it is expressed. It is also sensitive to technetium and permits it's the concentration within the cell across the cell membrane. This pilot study was carried out to test the feasibility of imaging of the transplanted heart with radioactive ^{99m}Tc following gene transfer with the Ad-NIS virus.

Methods

Inbred Lewis rats were used for syngeneic heterotopic cardiac transplantation. Donor rat hearts were perfused ex vivo for 30 minutes prior to transplantation with either plain University of Wisconsin (UW) solution (n=6) or UW solution with 10^9 pfu/ml of adenovirus expressing hNIS (Ad-NIS; n=6). On post-operative day (POD) 5 all animals underwent micro-SPECT/CT imaging of the donor hearts after tail vein injection of ^{99m}Tc.

Results

In the animals imaged with the use of ^{99m}Tc radioprobe, all hearts perfused with Ad-NIS demonstrated higher signal intensity compared to the hearts perfused with UW solution in which little or no signal was to be found in the abdomen in the region of the transplanted heart. Sequential imaging of the same animal over 60 minutes following injection of ^{99m}Tc did not reveal any appreciable differences

in signal intensities between 5, 25 and 45 minutes following injection of the isotope.

Conclusions

NIS can be used as a reporter gene in gene therapy to the transplanted heart. The expression of this reporter gene may be monitored non-invasively by serial radioisotope SPECT imaging. Sequential real-time detection and quantification of reporter gene expression is therefore feasible in cardiac transplantation.

3.1 INTRODUCTION

^{99m}Tc was first discovered in 1938 as a product of the degradation of radioactive molybdenum. ^{99m}Tc is the nuclear isomer of technetium-99 and is one of the most commonly used radioisotopes in nuclear medicine. Safety profile in its clinical applications has therefore been studied and documented. Technetium-99m when used as a radioactive tracer can be detected in the body by gamma cameras. It is well suited to the role because it emits readily detectable gamma rays that can be imaged by SPECT scanners, and its half-life for gamma emission is 6.0058 hours. The short physical half-life of the isotope and its biological half-life of 24 hours allows for scanning procedures that collect data rapidly but keep total patient radiation exposure low.

Sodium iodide symporter (NIS) is an integral plasma membrane glycoprotein that mediates active iodide transport into the thyroid follicular cells. In addition to iodide, it also facilitates the transport of technetium into the cells in which it is expressed on the membrane. It should therefore be possible to use NIS as an imaging reporter gene to monitor the expression profile of the transgene with the use of ^{99m}Tc and nuclear imaging. The absence of NIS on cardiomyocytes makes it an interesting myocardial reporter gene. The transduction of NIS encoded into an adenovirus did not cause myocardial injury or dysfunction in previous *in vivo* studies [162]. Lee et al. injected rat hearts with either Ad-EGFP (control) or Ad-EGFP-NIS and performed serial echocardiographic assessments of LV dimensions, heart rate, ejection fraction and fractional shortening (FS) to look for any evidence of myocardial injury on days before and days 4 and 9 following

Adenoviral NIS gene transfer. Separate rats underwent serial measurements of serum CK, myocardial myeloperoxidase assays and microscopic assessment of inflammation. Serial echocardiography revealed no difference in heart rate, LV dimensions, or functional parameters between Ad-EGFP-NIS and Ad-EGFP groups at any given time. Mild reductions in LVEF and LVFS by day 9 compared with baseline were similar for both Ad-EGFP and Ad-EGFP-NIS groups. Serial serum CK and myocardial myeloperoxidase activities were not elevated in either group. Histology revealed similar mild inflammatory cell infiltration restricted to the injection site for both groups. These data suggest that NIS gene transfer by itself does not have deleterious effects on myocardial function.

This pilot study was carried out to test the feasibility of imaging of the transplanted heart with ^{99m}Tc following gene transfer with the Ad-NIS virus. The feasibility of SPECT imaging after intravenous injections of ^{99m}Tc was tested in a rat model of syngenic heterotopic cardiac transplantation. Comparisons were made between donor hearts perfused with Ad-NIS and hearts perfused with UW solution prior to transplantation.

3.2 MATERIALS AND METHODS

Animals

Inbred male Lewis rats (270-350 g) were used as donors and recipients for syngeneic transplants. Procedures and handling of animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Mayo Clinic and Foundation in compliance with "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (National Institutes of Health publication No. 86-23, revised 1985).

Adenoviral vector

A first generation E1A deleted (replication defective) serotype 5 adenovirus encoding for human Sodium Iodide Symporter (hNIS) under the control of the cytomegalovirus promoter was used in this study (Ad-CMV-hNIS). The recombinant virus was propagated in transformed human embryonic kidney carcinoma cells ("293 cells"), which constitutively express E1 proteins; isolated and purified and stored at –70°C in a buffered solution of 10% glycerol until use. Viral titers were determined by means of plaque assay. The plaque assay method has been the most established/traditional method of titering virus, giving the user an accurate and consistent determination of the concentration of infectious viral particles. The assay is carried out using Human Embryonic Kidney (293) cells. Serial ten-fold dilutions in 2% DMEM were performed for each virus to be titered. After a 90-minute infection time, the viral solution was removed and the cells

layered with a 1% agarose mix to allow for the generation of mature plaques. Plaques were counted after a period of 10 days and the titer expressed in plaque forming units per mL (pfu/ml).

Donor operation

After induction of anaesthesia, (pentobarbital sodium 70 mg/kg administered intraperitoneally), the donor rat was intubated and mechanically ventilated (model 683; Harvard Apparatus Inc, South Natick, Mass; tidal volume: 10 mL/kg, respiratory rate: 60 breaths/min). A median sternotomy was performed to expose the heart. After injection of 200 U of aqueous heparin into the inferior vena cava, the innominate artery was cannulated with a 24-gauge cannula, and the venacavae and pulmonary veins were ligated en bloc with 6-0 silk. The aorta was clamped distal to the cannula, and the heart was arrested with an infusion of cold University of Wisconsin solution (UWS) into the aortic root through the indwelling cannula (flow, 0.44 mL/min; duration, 5 minutes). After harvesting, the heart was stored in the same cardioplegic solution at 4°C.

***Ex vivo* Perfusion of the explanted heart**

In this first series of transplants, donor hearts were perfused for 30 minutes with either Ad-NIS or UW solution at 4°C prior to being transplanted into the recipient as described. The efficiency of adenovirus-mediated gene transfer was evaluated in 2 groups (n = 6 in UW group and n=6 in Ad-NIS group). Rats were randomly assigned to each group. In the two groups perfused with the viral vector, the virus was not flushed out before performing the surgical procedure. In group Ad-NIS, 5 mL of UW solution containing 1×10^9 PFU/mL of Ad-NIS was circulated

through the coronary vasculature of the donor organ for 30 minutes by means of a peristaltic pump (Rainin, Emeryville, CA). UW solution without the virus was used for donor heart perfusion in the UW group. The solution was infused into the donor organ through a cannula inserted into the aortic root and was collected by a 14-gauge catheter placed into the pulmonary artery. Both catheters were connected by means of polyvinyl chloride tubing to the vial containing the viral solution. The flow rate was 0.75 mL/min. Earlier experiments in this lab have determined the aortic root pressure to be in the region of 40-50 mm Hg with this flow rate. During the perfusion period, the container with the heart and the vial with the vector were kept on ice, and the temperatures of both solutions did not exceed 10°C. Entrapment of air in the perfusion apparatus was avoided by keeping the heart vertical during perfusion.

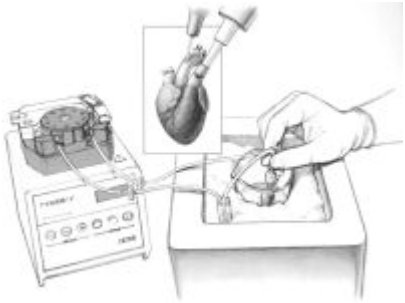


Illustration of the gene delivery system used in the current study

The solution containing the adenoviral vector was perfused from a vial through the vasculature of the donor heart and back to the vial by means of a peristaltic pump *(left)*. The container with the heart immersed in UW solution and the vial with the viral solution were kept on ice during the circulation time. The *inset* depicts the inflow and outflow catheters placed in the aorta and pulmonary artery, respectively.

Recipient operation

Heterotopic abdominal heart transplantation was performed by using standard microsurgical techniques as described previously[155]. Animals were anesthetized by administration of intra-peritoneal pentobarbital (70 mg/kg). The donor hearts were transplanted into the recipients by end-to-side anastomoses of the aorta and the pulmonary artery to the abdominal aorta and inferior vena cava, respectively, by using 10-0 monofilament sutures. During surgery, the heart was wrapped in gauze and kept cold by use of topical ice-cold UW solution. At reperfusion, appearance and contractility of each graft was conformed to be satisfactory. All rats received analgesia postoperatively (Buprenorphine dose subcutaneously) and recovered with oxygen in a warm environment. Function of the graft was checked daily by palpation of the beating transplanted heart.

SPECT Imaging with ^{99m}Tc radio probe

All these recipients were imaged at day 5 using ^{99m}Tc with the SPECT/CT imaging system.

The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (70 mg/kg) for the imaging procedure. 1000μCi of ^{99m}Tc was injected intravenously through the tail vein and images obtained over the next hour starting with 5 minutes after the injection. CT scans of the abdomen were obtained in the intervening period to enable visualization of the heart in the abdomen. Gamma camera imaging was performed to visualize the transplanted hearts *in vivo*. Protocols for imaging have been described in the chapter 4 and in Appendix A and B. Whole-body image was not feasible due to the size of the rats. The region of interest was therefore limited to an area from the level of the diaphragm down to the urinary bladder. Images were obtained and stored using custom-designed software.

3.3 RESULTS

There were no deaths at any time point during the protocol. All transplanted hearts were beating and grossly normal with no areas of obvious ischaemia at the time of harvest.

Under the gamma camera, all transplanted hearts perfused with Ad-NIS demonstrated a higher signal intensity compared to the hearts perfused with UW solution in which little or no signal was to be found in the abdomen in the region of the transplanted heart. (Image 1) Sequential imaging of the same animal over 60 minutes following injection of ^{99m}Tc did not reveal any appreciable differences in signal intensities between 5, 25 and 45 minutes following injection of the isotope. (Image 2) Quantification of the percentage of injected dose taken up was not possible in this series due to difficulties drawing up normograms equating definite signal intensities with radioactivity counts. This drawback was corrected in the second series of transplants using radioiodine to probe the presence of NIS. (Chapter 4)

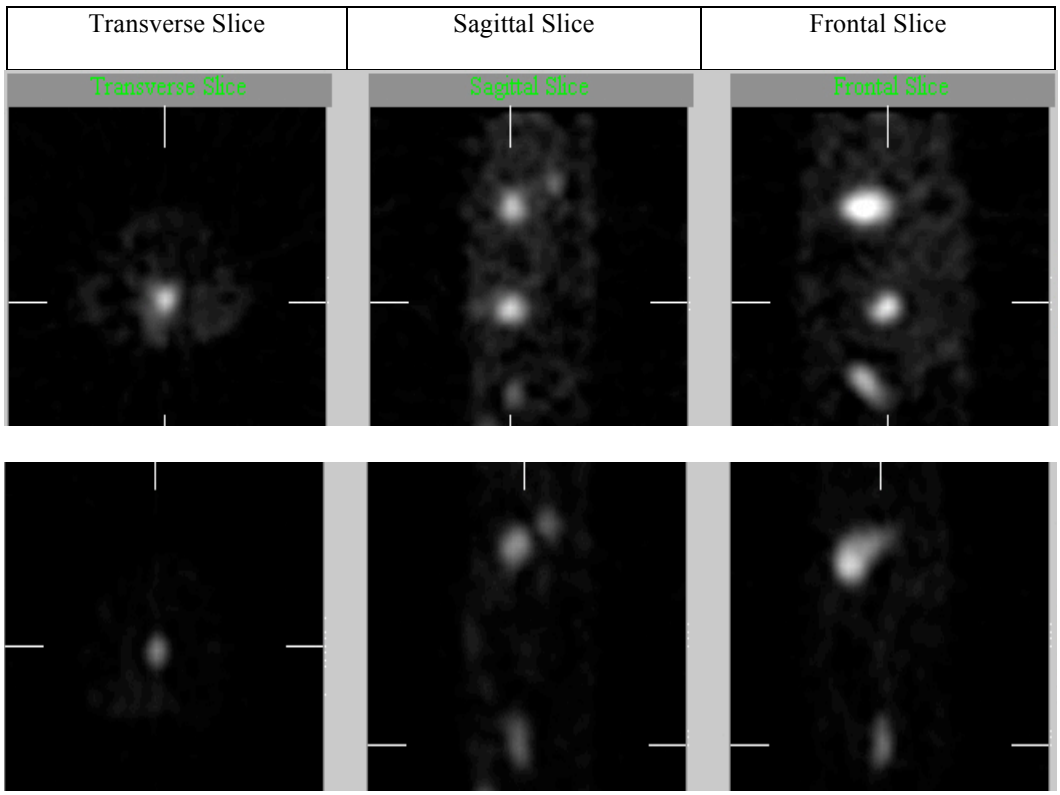


Image 1

SPECT imaging of the transplanted heart 20 minutes after tail vein injection with ^{99m}Tc five days after transplantation. Top panel shows an Ad-NIS perfused heart and the bottom panel shows a heart perfused with plain UW solution.

The “hot spot” in the top panel is due to the concentration of technetium by the heart perused with Ad-NIS. The “hot spot” above the transplanted heart corresponds to the radioisotope concentration in the stomach due to the inherent presence of NIS in the gastric mucosa.

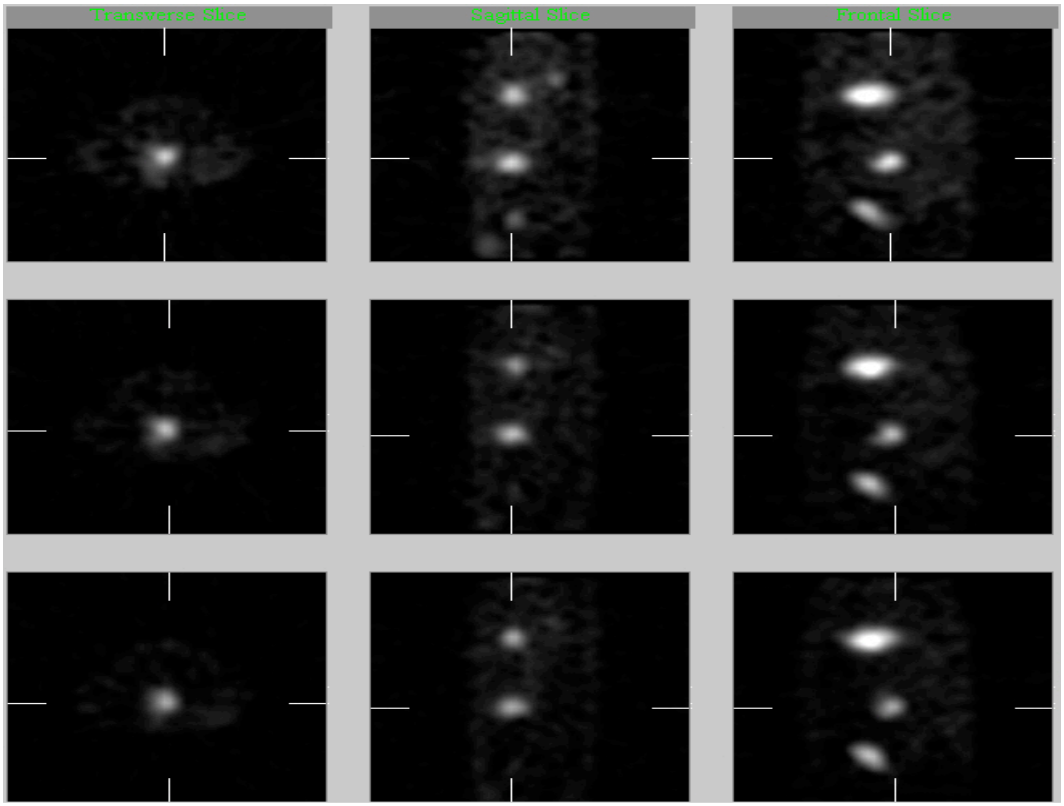


Image 2

SPECT imaging of the transplanted heart 5 minutes (top panel), 25 minutes (middle panel) and 45 minutes (bottom panel) after tail vein injection with ^{99m}Tc five days after transplantation. No appreciable difference was found in the intensity of the images obtained at these three time points.

3.4 DISCUSSION

Although several studies have mapped cardiac transgene expression in time and uniformity, a technique for *in vivo* imaging and quantification of gene transduction has hitherto been absent. The use of adenovirus coding for hNIS opens up the possibility of sequential real-time imaging of gene transduction with currently available SPECT imaging facilities.

This preliminary set of experiments with the use of ^{99m}Tc demonstrated the feasibility of sequential non-invasive monitoring of gene expression in cardiac transplantation. To our knowledge, this is the first study of this nature to be carried out in cardiac transplantation.

In this study the heterotopic hearts perfused with a solution containing Ad-NIS presented a significantly higher uptake of technitium with respect to the control group (UW only) 5 days after the *ex vivo* perfusion. All transplanted hearts showed an image intensity peak on POD 5 with a radioiodine uptake that was still significantly higher than in the control group. Animals were imaged on day 5 based on previous experiments with gene transfer and knowledge of the kinetics of adenovirus serotype 5.

Finding methods to monitor the profile of gene expression over time is a challenge. Expression of cell-associated transgenes can be monitored by immune-histochemical analysis of tissue biopsies obtained from the site of gene transfer [163]. Tissue sampling is therefore an unsatisfactory method to assess the efficiency

of gene delivery because the biopsy that is subjected to analysis is essentially a random sample from an area of non-uniform gene delivery. Also, there is a limit to the number of times that a given tissue can be biopsied, and for certain tissues, such as the heart, tissue biopsy is associated with a high risk of mortality.

Our data demonstrate that hNIS is an excellent reporter gene for the transplanted heart. It has also been demonstrated by other groups that myocardial NIS gene imaging does not cause significant myocardial injury or affect cardiac function other than effects due to adenoviral vector-associated host response [162]. In addition the use of a species-specific isoform such as hNIS for humans may help to circumvent any problem caused by the immunogenicity of transgene products. Despite its limitations we chose to use a recombinant adenoviral vector, with a deleted E1 region to prevent replication *in vivo*, because of the easy production, our experience with it and the high transduction efficiency obtained with this vector [55, 64, 87].

In this series, there were difficulties with continued use of ^{99m}Tc to image the transplanted heart due to a global shortage of molybdenum. Available ^{99m}Tc was therefore channeled to clinical applications in this institution. Although the feasibility of nuclear imaging was proven with these experiments it was thought preferable to explore a model in which gene expression could be quantified. In addition, we wanted to explore the possibility of defining the heart borders anatomically with CT scanning carried out at the same time as SPECT imaging. Further experiments with the NIS gene construct were therefore performed with ^{123}I and SPECT/CT imaging as has been outlined in the next chapters.

Conclusions

This series of experiments proves the feasibility of gamma camera imaging of gene transduction with the hNIS symporter in the transplanted heart.

Chapter 4.

NUCLEAR IMAGING OF GENE TRANSDUCTION IN THE TRANSPLANTED HEART WITH SODIUM IODIDE SYMPORTER (hNIS) AND ^{123}I

Brief abstract

The feasibility of SPECT imaging after intravenous injections of ^{123}I radiolabelled ligand was tested in a rat model of syngenic heterotopic cardiac transplantation. Comparisons were made between donor hearts perfused with Ad-NIS and hearts perfused with Ad-Null and UW solution prior to transplantation.

ABSTRACT

Background

We evaluated the feasibility of non-invasive micro-single photon emission computed tomography/computed tomography (micro-SPECT/CT) imaging and quantification of cardiac gene expression following sodium iodide symporter (hNIS) gene transfer in cardiac transplantation.

Methods

Donor rat hearts were perfused *ex vivo* with adenovirus expressing hNIS (Ad-hNIS), Ad-Null or University of Wisconsin (UW) solution prior to heterotopic transplantation into syngeneic recipients. In the first group of recipients, imaging of the transplanted hearts with micro-SPECT/CT on day 5 was followed by immediate explant of the organs for *ex vivo* analyses. Radioactivity counts in the explanted hearts were obtained *ex vivo* and expressed as a percentage of the injected dose per gram of tissue (%ID/g). Intensities of the SPECT images of the transplanted hearts were quantified and converted to radioactive counts using a standard equation. The second group of recipients was imaged sequentially following injections of ^{123}I on days 2 to 14 following transplantation.

Results

Higher *ex vivo* radioiodine counts were noted in the hearts perfused with Ad-hNIS (1.04±0.2) compared to either the UW group (0.31±0.11, $p<0.001$) or the Ad-Null group (0.32±0.08, $p<0.001$) (%ID/g). Image intensity in the Ad-NIS group was also significantly higher than in the UW group (0.4±0.03, $p=0.003$) or

the Ad-Null group (0.5±0.1, $p<0.05$) (%ID/g). Sequential imaging of Ad-NIS-perfused hearts between post-operative days 2 and 14 revealed peak image intensity at day 5. Overall, image intensities correlated with *ex vivo* counts of radioactivity ($\rho=0.74$, $p<0.05$).

Conclusions

These data demonstrate that hNIS gene transfer permits sequential real-time detection and quantification of reporter gene expression in the transplanted heart with micro-SPECT/CT imaging.

Key words

Gene therapy, heart transplantation, sodium iodide symporter, single photon emission computed tomography (SPECT), molecular imaging

4.1 INTRODUCTION

The concept of transduction of therapeutic genes that could alter the course of pathological processes at a cellular level is promising. In the context of myocardial gene therapy, confirming uniform, persistent and reproducible transgene expression is essential prior to assessment of the effectiveness of therapeutic genes by functional studies. Laboratory studies in gene therapy aimed at affecting pathological processes in cardiac transplantation have suffered from the absence of a reliable non-invasive reporter system to confirm and monitor the location and magnitude of gene expression over time. Non-invasive imaging of reporter genes offers the advantage of sequential imaging of the same animal with the possibility of using fewer animals in pre-clinical studies as well as a direct applicability to humans.

The sodium/iodide symporter (hNIS) present on the thyroid follicular cell facilitates the concentration of iodine into the cell for thyroid hormone synthesis. The presence of the symporter on the thyroid follicular cell but not on the cardiomyocytes or the vascular endothelium makes it an attractive myocardial reporter gene product. Radioiodine concentrated by the expressed symporter could be detected by nuclear imaging techniques to localize and quantify gene expression. Several studies in tumour models of gene transfer have validated the effectiveness of hNIS as a non-invasive reporter gene [135, 136, 164, 165]. In addition, *in vitro* analyses have demonstrated the absence of any myocardial injury or dysfunction after Ad-NIS gene transduction [162].

Although image analysis to quantify radioligand uptake is representative of NIS gene transduction in the heart [61, 62, 166] accurate mapping of the margins of the organ is essential to ensure the semi-quantitative nature of this technique. Recent reports using NIS reporter system have indicated more accurate quantification of gene expression with the use of computed tomography (CT) co-registered with micro-single photon emission computed tomography (SPECT) than with the use of planar or micro-SPECT imaging alone due to better tomographic representation facilitating image mapping of the transduced organ [167]. In this study, we evaluated the feasibility of non-invasive micro-SPECT/computed tomography (CT) imaging with precisely co-registered axial CT scans to quantify of gene expression after hNIS gene transfer.

4.2 MATERIALS AND METHODS

Inbred male Lewis rats (225-350 g, Harlan®, IN) were used as donors and recipients for syngeneic heterotopic heart transplants. Procedures and handling of animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Mayo Clinic and Foundation in compliance with guidelines published by the National Institutes of Health (National Institutes of Health publication No. 86-23, revised 1985).

Production of Recombinant Adenovirus

A replication-deficient human recombinant adenovirus serotype 5 (Ad) construct containing human NIS under the control of the CMV promoter (Ad5/CMV/hNIS) was produced in collaboration with the Mayo Clinic Vector Production Facility using previously described methods.[168] Following plaque purification, the recombinant adenovirus Ad5/CMV/NIS was expanded in 293 cells and purified by banding on Cesium chloride density gradients, followed by dialysis. Purified virus was diluted in cold University of Wisconsin (UW) solution to a concentration of 10^9 pfu/ml just prior to *ex vivo* perfusion of donor hearts.

Donor operation

After anesthesia (intraperitoneal pentobarbital sodium 70 mg/kg), the donor rat was intubated through an open tracheostomy and mechanically ventilated (model 683; Harvard Apparatus Inc, South Natick, MA; tidal volume 10ml/kg;

respiratory rate 60/minute). A median sternotomy was performed and 200 U of aqueous heparin injected into the inferior vena cava. The innominate artery was cannulated with a 24-gauge cannula, and the venae cavae and pulmonary veins were ligated en bloc with 6-0 silk. The aorta was clamped distal to the cannula, and the heart was arrested with an infusion of cold UW solution into the aortic root through the indwelling cannula (flow, 0.73 mL/min for 5 minutes). After harvesting, the heart was stored in UW solution at 4°C.

Experimental groups and Gene Transfer

In the first set of experiments, the donor hearts were perfused with 10^9 pfu/ml of Ad-hNIS in UW solution (n=6), 10^9 pfu/ml of Ad-Null in UW solution (n=6) or UW solution alone (n=3) for 30 minutes at 4°C using a hypothermic *ex vivo* perfusion system as previously described [65]. All recipients in this set were sacrificed following imaging on day 5.

In the second set of experiments, donor hearts were perfused with 10^9 pfu/ml of either Ad-hNIS (n=3) or Ad-Null (n=3) diluted in 5 ml of UW solution under similar conditions. These recipients were imaged sequentially on days 2, 5, 9 and 14 following gene transfer.

Recipient operation

Following anesthesia (intraperitoneally pentobarbital sodium 70 mg/kg) a midline laparotomy was performed and the abdominal aorta and the inferior vena cava exposed below the origin of the renal vessels. The donor hearts were transplanted

into the recipients by end-to-side anastomoses of the aorta and the pulmonary artery to the abdominal aorta and inferior vena cava, respectively, using 10-0 monofilament sutures.[155] During surgery, the heart was wrapped in wet gauze and cooled with topical ice-cold UW solution. All rats received analgesia postoperatively and recovered with oxygen in a warm environment. Function of the graft was checked daily by palpation of the beating transplanted heart.

Micro-SPECT/CT Acquisition Protocol

All animals were imaged under sedation (intraperitoneal pentobarbital 50mg/kg). 37 MBq of ^{123}I was injected into the tail vein and micro-SPECT/CT imaging commenced after 20 minutes. Syringe activity was measured prior to and post-injection to quantify the injected dose accurately. Micro-SPECT images were obtained under a high-resolution gamma camera (X-SPECT, Gamma Medica-Ideas Inc., CA) with a low energy high-resolution parallel hole collimator with 64 projections at a rate of 10s per projection and an acquisition time of 13m 46s. CT images were obtained under a circular orbit at a thickness of 50μM per slice under the same scanner without moving the animal in between. This ensured accurate co-registration of the axial CT images with the micro-SPECT maps.

CT images were reconstructed using a modified Feldkamp (cone-beam filtered backprojection) reconstruction algorithm into a 512^3 matrix with a voxel sizeof 0.1557 mm. This results in a field of view of 7.97 cm x 7.97 cm. The CT system is capable of producing images with a resolution of 50μM; however with this reconstruction setting the voxel size limits the CT resolution to 0.3114 mm. The SPECT images were reconstructed using a Filtered Backprojection algorithm into

a $80mm^3$ matrix size with a voxel size of 1.5 mm. This resulted in a field of view of 120mm x 120mm. The resolution of the SPECT system is limited by the collimater resolution with parallel-hole collimators and is approximately 3-4mm.

Image Analysis and *In Vitro* Quantification

SPECT and CT images were stored and pixel-intensity of the images was quantified as described in earlier studies.[167] Briefly, the regions of interest (ROI) were defined manually around the heart shadows on the reconstructed axial tomographic images in all sections in which the heart was visualized to create a volume of interest (VOI). Quantification was performed on the co-registered micro-SPECT images (Fig 1). Cumulative pixel counts within the defined region of interest (ROI) were converted to activity using a conversion factor of 1.63 x 10^{-7} MBq/measured count derived from our previous studies[167]. This conversion of pixels into counts of radioactivity by using equations derived from scanning a standard containing a known quantity of radioactive ^{123}I. Values expressed as a percentage of the injected dose per gram of heart tissue (%ID/g). All activity measurements were corrected for decay of the isotope (^{123}I half-life = 13.27 hours) and all counts of radioactivity mentioned later in this manuscript refer to 'zero time', i.e corrected to the time at which the rats were injected with radio-iodine. An independent observer blind to the origin of the images obtained measurements.

In the first set of experiments, all rats (n=15) were sacrificed following imaging of the transplanted organ on day 5. In the second series of experiments, rats (n=6)

were imaged on post-operative days 2, 5, 9 and 14 and sacrificed on day 14. Transplanted hearts were recovered following sacrifice, flushed clear of blood and weighed. Radioactivity in the hearts was measured using a National Institute of Standards and Technology (NIST)-calibrated dose calibrator. Values were expressed as a percentage of the injected dose per gram of tissue (%ID/g) after correcting for radioiodine decay to the time of image acquisition ($t_{1/2}$ of ^{123}I=13.2 h). All values thus relate to counts of radioactivity at the commencement of SPECT imaging 20 minutes following injection of radioiodine.

Histology –Rejection severity

Following explant of the transplanted hearts, mid-ventricular sections were cut, embedded in OCT compound (Miles Laboratories, Elkhart, IN) and snap-frozen in liquid nitrogen–cooled 2-methylbutane for histologic evaluations. Adjacent sections of the ventricle was fixed in formalin and embedded in paraffin. Sections were cut and mounted on slides for staining with haematoxylin and eosin. The remainder of the ventricle was snap-frozen in liquid nitrogen before being stored at -70˚C.

Adjacent slides were cut and stained with hematoxylin and eosin for routine histopathologic examination. An experienced cardiac pathologist blind to the origin of the slides graded inflammation and ischemic damage. Slides were scored on a scale comparable with the working formulation for cardiac rejection[169]. Sections were examined and graded for ischemic injury based on the degree of myocardial damage noted as a percentage of the total slide by area.

Degree of myocardial coagulative necrosis, extent of cellular infiltrates and myocyte vacuolization were estimated on haematoxylin and eosin-stained preparations of the myocardium form explanted hearts.

The following scale was used: 0, no ischemic damage; 1- less than 5% of the area of the section; 2- between 5% and 20% of the area; 3- between 20% and 40% of the area; and 4- more than 40% of the area.

Statistics

All results were expressed as mean ±SD. In the first set of transplants, comparisons of transduction rates between the UW, Ad-Null and Ad-NIS groups were drawn by the non-parametric Kruskal-Wallis test. The Wilcoxon test was used for comparison of transduction rates between pairs of groups. Paired *t* tests were used to compare corresponding measurements between the two groups of animals imaged sequentially in the second set of experiments. Friedman's test was used to identify significant differences in image intensity within Ad-NIS and Ad-Null groups over 14 days. Correlation between counts of explanted hearts and intensity of images obtained on the same day was assessed by Spearman correlation. In all analyses, $p < 0.05$ was considered statistically significant.

4.3 RESULTS

Counts of radioactivity in the explanted hearts and correlation of *in vivo* counts with *ex vivo* radioiodine uptake

In the initial set of experiments, all recipients were sacrificed following the imaging protocol on day 5. Transplanted hearts were recovered and radioactivity counts were measured using a dose calibrator. Intensity of images obtained of each transplanted heart was quantified as described earlier. This enabled a comparison between radioactivity trapped by the organ with the intensity of captured images. We hypothesized that the organs perfused with Ad-NIS would trap a higher fraction of injected radioiodine due to the expression of NIS on the cell membrane.

In this set of recipients, significantly higher *in vitro* radioactive counts were noted in the hearts perfused with Ad-hNIS (1.04±0.2) compared to either the UW group (0.31±0.11, $p<0.001$) or the Ad-Null group (0.32±0.08, $p<0.001$) (mean ±SD; %ID/g). Image intensity in the Ad-NIS group (0.9±0.2) was also significantly higher than in the UW group (0.4±0.03, $p=0.003$) or the Ad-Null group (0.5±0.14, $p<0.05$) (mean ±SD; %ID/g; Fig 2). No differences were noted in radioactivity levels or image intensities between the UW and Ad-Null perfused hearts ($p=0.714$; Table 1). This comparison between the Ad-Null and UW-perfused hearts suggests that differences in radioiodine uptake between the groups were not due to an inflammatory response following adenoviral transduction. The higher image intensity of micro-SPECT images in the Ad-NIS

group was reflected in counts of radioactivity in the explanted hearts *in vitro*. Overall, image intensities correlated positively with *ex vivo* counts of radioactivity ($\rho=0.74$, $p<0.05$; Fig 3).

Sequential ^{123}I scintigraphy and quantification of image intensity in the groups

In the second set of recipients, comparisons were drawn between Ad-NIS and Ad-Null perfused transplanted hearts between the second and 14th days post-transplantation. Sequential imaging of recipients was carried out to visualize variations in image intensity in Ad-NIS-perfused hearts consistent with gene expression following serotype 5 adenoviral transduction. Cumulative pixel counts of images were converted to measures of radioactivity as described. Comparative image intensities on days 2, 5, 9 and 14 were 0.59±0.14, 1.08±0.15, 0.87±0.19 and 0.53±0.27 in the Ad-hNIS perfused hearts and 0.4±0.08, 0.45±0.12, 0.47±0.09 and 0.44±0.09 in the Ad-Null perfused hearts respectively (%ID/g; mean ±SD; Fig 4). As expected, peak image intensity was noted on day 5 in Ad-NIS perfused hearts. Image intensities measured on days 5 and 9 in the Ad-NIS group were significantly higher than those in the Ad-Null group were on the same days. Image intensities on days 5 and 9 were higher than at days 2 and 14 in the hearts transduced with Ad-NIS. A peak in image intensity on day 5 followed by a fall to background levels by 14 days reflects the kinetics of the serotype-5 recombinant adenovirus in the transplanted heart. This also suggests that radioiodine trapping was due to the expression of the symporter rather than non-specific uptake. No differences were noted in values obtained from Ad-Null

perfused hearts on sequential imaging over time.

Grading of ischaemic injury of the transplanted hearts

Ischaemic injury was graded 0 to 4 (4 = maximal damage) by an experienced pathologist blind to the origin of the tissue. The mean grades were as follows:

Group	**N**	**Mean ± SD**	**Median**	**Range**
Ad-NIS	6	1.17 ± 0.41	1	(1,2)
Ad-Null	6	1.50 ± 0.84	1	(1,3)
UW	3	1.67 ± 1.15	1	(1,3)

Fisher's Exact Test to detect a difference in proportions between any two groups returned a p-value of 0.07. Further evaluation testing for a trend among each set of pairwise groups, as well as dichotomizing grade into low damage and high damage groups did not reveal a difference between the groups.

4.4 DISCUSSION

To our knowledge, this is the first report of the use of a reporter gene permitting non-invasive nuclear imaging of gene transduction in cardiac transplantation. In this study, radioiodine uptake by the heterotopic hearts in the hNIS group was significantly higher than in the Ad-Null group. In addition, serial imaging of the same animals over 14 days revealed a peak in radioiodine uptake by the transplanted heart at 5 days followed by a decline to baseline levels at 14 days (Fig 4). This is consistent with the kinetics of the adenovirus serotype 5 in the transplanted heart[170-172]. In addition, the absence of any difference in radioiodine trapping between Ad-Null and UW-perfused hearts suggests that radioisotope uptake was not due to the inflammatory response caused by the adenovirus but specific to the presence of the symporter expressed in transplanted hearts. These data together suggest that NIS gene transfer permits the imaging of gene transfer in real-time with the use of SPECT/CT. In addition, sequential imaging of the same animal to track decaying gene expression is feasible with this technique.

The use of gene therapy for cardiovascular disorders requires careful selection of genes with careful consideration of the pathology being targeted. In addition, studies assessing therapeutic effect of functional genes assume reliable and reproducible transfection of the target organ. These factors are addressed by the use of reporter genes. Nuclear imaging of reporter genes presents the opportunity to assess levels of transgene expression in real-time without sacrificing the animal, thereby allowing completion of pre-clinical studies with fewer animals. Thymidine kinase, hNIS and norepinephrine transporter (NET) gene transfer all

permit concentration of radioisotopes that could be imaged non-invasively [60, 119, 127]. Potentially, the site and density of gene expression can be quantified with the use of PET or SPECT imaging. However, the potential immunogenicity of the thymidine kinase gene product and the inherent presence of NET in the human heart are limitations to the use of these reporter genes in cardiac transplantation. The presence of hNIS on mammalian thyroid follicular cells makes it attractive due to the potentially attenuated immune response to the gene product. Although PET imaging of gene transduction is feasible,[104, 119, 166] semi-quantitative and allows tomographic reconstruction of transduction sites in the myocardium, PET scanners are not as widely available and require the production of expensive tracers with on-site cyclotrons. These problems are avoided by the use of SPECT to image NIS expression.

Limitations of molecular imaging studies relate to the selection of established clinical imaging modalities to visualize gene expression. Head to head comparisons of image quantification have shown the use of co-registered axial CT images to map the regions of interest to be more accurate and less variable than micro-SPECT or planar imaging alone in predicting *ex vivo* radioactivity counts.[167] Anatomical detail offered by CT images ensures a more precise definition of organ boundaries compared to SPECT images. However, small animal scanners are limited in their resolution and the use of clinical grade CT scans to map the regions of interest as well as a translation of this system to the orthotopic heart transplant model is likely to produce images that ensure a more accurate quantification of image intensity and reduce the variability within groups noted in this study. Use of clinical-grade SPECT/CT equipment would also

permit precise quantification of the cardiac dimensions including cavity size and dimensions of the myocardium, the actual site of gene transduction. This was not possible with micro-SPECT/CT. In addition, quantification of images obtained at a definite time point was based on the assumption that radioiodine uptake by all NIS expressing cells is uniform in time. In this study, the stomach demonstrated a 'hot-spot' in all images due to the inherent presence of the iodide symporter in the gastric mucosa. The presence of background levels of radioiodine in the hearts not perfused with the hNIS gene could be explained by the presence of the radioisotope in the circulating blood as well as some non-specific uptake in the extracellular regions of the transplanted heart.

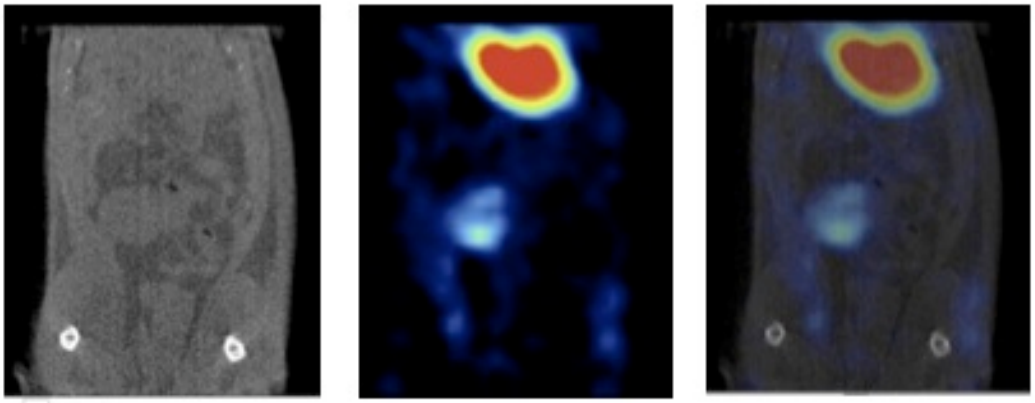
Although promoter-driven NIS expression is functional, radioiodide trapping is transient due to the absence of organification in non-thyroidal tissue. In non-thyroidal cancer, NIS suicide-gene transfer has been used to facilitate concentration of ^{131}I that is cytolytic to tumor cells. In the transplanted heart however, NIS is used merely as a reporter gene and no cytolytic effects are desired following radiotracer uptake. In this study, quantification of gene expression was validated with ^{123}I without the use of longer acting radiotracers. ^{123}I is commercially available and routinely used in clinical imaging studies. Rapid efflux of the tracer from the cardiomyocyte and a short half-life of this isotope enable repeated imaging of the transplanted organ. In addition, non-thyroidal NIS expression is unaffected by levels of thyroid stimulating hormone (TSH). These factors make this an attractive cardiac reporter gene system.

Conclusions

The use of hNIS reporter gene facilitates the non-invasive imaging of gene transduction in cardiac transplantation. Molecular imaging using SPECT permits sequential imaging of the subject over time. In addition, the decay of the gene probe can be monitored over time to determine feasibility of the use of specific vectors or therapeutic genes to target a specific pathology following transplantation. Expertise gained with cardiac SPECT imaging and the radioiodine kinetics in humans ensures direct applicability of this concept to clinical trials of gene transfer.

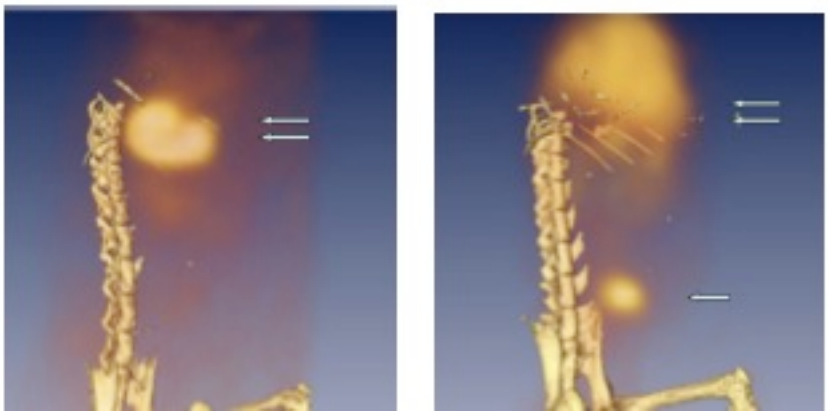
4.5 FIGURES AND TABLE

Figure 1



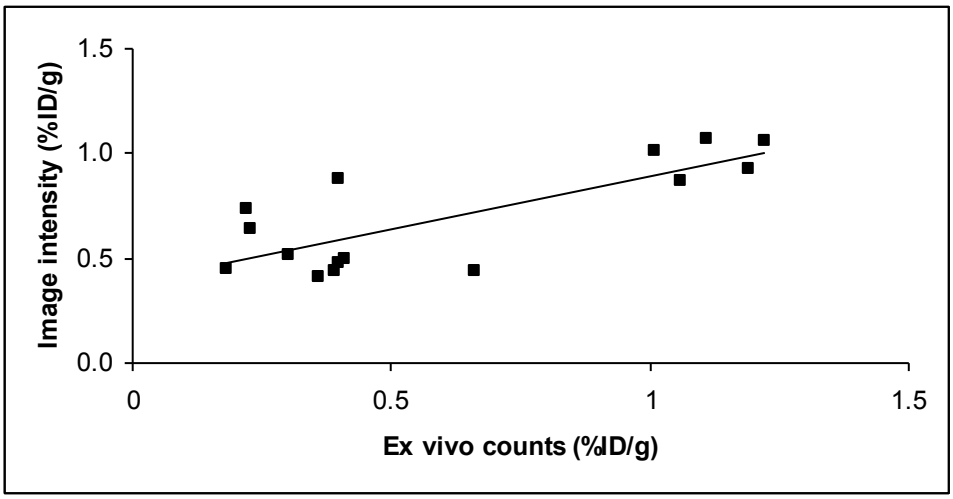
Antero-posterior view of the Abdomen showing CT scan (left), micro-SPECT (middle) and co-localization of the transplanted heart on superimposed micro-SPECT/CT images (right). The ‘hot-spot’ on the SPECT corresponds with the position of the Ad-NIS perfused transplanted heart noted on the CT. The ‘hot spot’ above the transplanted heart corresponds to the concentration of radioiodine in the stomach due to the inherent presence of NIS on the gastric mucosa.

Figure 2



Fused micro-SPECT/CT 3D reconstructed images of the recipient abdomen 5 days after gene transfer with either Ad-Null (left) or Ad-NIS (right). Higher radioiodine uptake by the Ad-NIS perfused heart (single arrow) is demonstrated as a hot spot. This is not seen in the Ad-Null perfused heart. Natural gastric mucosal uptake of radioiodine is seen as a ‘hot spot’ in both animals (double arrow).

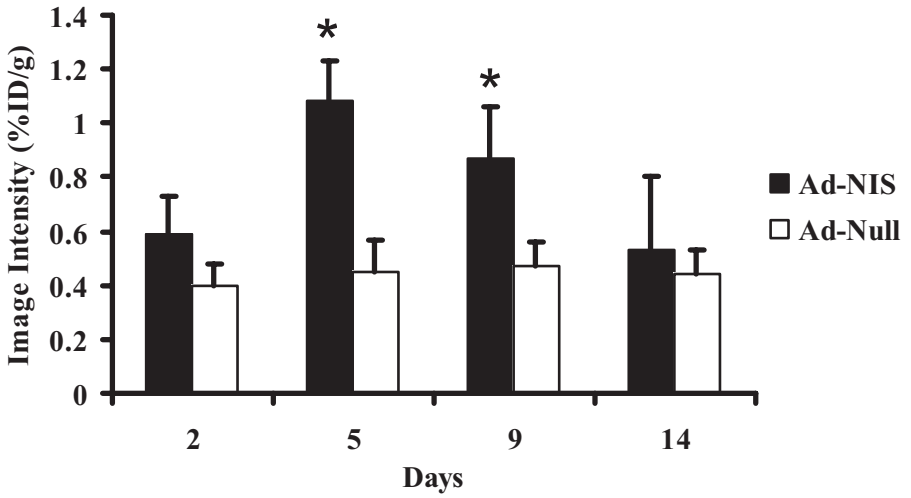
Figure 3



Comparison of radioiodine uptake determined by *ex vivo* counting of transplanted hearts (explanted on day 5 following transplant) with image analysis. *Ex vivo* counts of radioactivity correlated positively with those obtained by image analysis (Spearman correlation $\rho=0.74$, $p<0.05$).

Figure 4

Days	2	5	9	14
Ad-NIS	0.59±0.14	1.08±0.15*	0.87±0.19*	0.53±0.27
Ad-Null	0.4±0.08	0.45±0.12	0.47±0.09	0.44±0.09



Quantification of image intensity on sequential imaging following gene transfer Ad-NIS perfused hearts (solid bars) demonstrated significantly higher image intensities than Ad-Null perfused hearts (thatched bars) on days 5 and 9 following gene transfer (%ID/g; $p<0.01$ and $P=0.03$ on days 5 and 9 respectively) Hearts perfused with Ad-Null did not show differences in radioiodine uptake when imaged over 14 days

Table 1

	UW group	**Ad-Null**	**Ad-NIS**
Ex vivo	0.31±0.11	0.32±0.08	1.04±0.2*
In Vivo	0.4±0.03	0.5±0.14	0.9±0.2*

Counts of radioactivity *in vivo* and *ex vivo* by software analysis of image intensity and dose calibrator respectively. Data are expressed as % of injected dose of ^{123}I per gram of heart tissue. *Ex vivo* and *in vivo* counts of radioactivity were significantly higher in the hearts infected with Ad-NIS (*$p<0.05$) when compared to either of the other groups.

Chapter 5

DISCUSSION OF THE THESIS

Transplantation presents a unique opportunity in that gene transfer can be achieved through *ex vivo* perfusion of the organ thereby limiting the complications of *in vivo* gene delivery. In addition, the obligate use of immunosuppression permits a more efficient and sustained gene expression due to the attenuation of the immunogenicity of viral vectors. Although several studies have mapped cardiac transgene expression in time and uniformity, a technique for *in vivo* imaging and quantification of gene transduction has hitherto not been described.

The technique of obtaining fused SPECT-CT images to provide anatomical correlation with the physiological process of radioligand uptake was authenticated. In this set of experiments using ^{123}I for SPECT imaging, the correlation of in vivo quantification techniques using the designed software with the radioactivity in the transplanted hearts was demonstrated. SPECT-CT imaging has been used in nuclear medicine for attenuation correction of SPECT emission and provides anatomical correlation. In the heterotopic cardiac transplant model, accurate localization of donor heart margins was necessary to reduce noise generated by non-specific radioisotope uptake by surrounding bowel during image quantification.

In summary therefore, it is important to understand processes underlying the post-translational fate of the symporter as well as the intracellular kinetics of the

radioligand prior to attempting prediction of gene transfer efficiency with signal intensity as determined by nuclear imaging techniques. It is also important to study the behavior of the symporter expressed *in vivo* following *ex vivo* gene transfer. NIS expression could potentially be influenced both by drugs and by cytokines [173, 174]. The presence of an altered cytokine milieu following clinical cardiac transplantation due to the pre-existent recipient pathology requiring transplantation, the presence of the allograft, the exposure to cardiopulmonary bypass and immunosuppressant drug-priming of the recipient is likely to influence NIS expression as well as radioisotope uptake and needs further investigation.

5.1 Limitations of NIS Gene expression and Imaging

The variability of NIS expression within groups could represent a disadvantage of the Ad5 vector system that is well recognized. Ad5 dependence on CAR to transfect cells could contribute to these variations.

Closer observation of the individual data points seems to suggest that at lower radioactivity levels, the software tends to overestimate the signal intensity. This reflects the limitations of the heterotopic model. The transplanted heart is situated in the abdomen and some signal attenuation is inevitable. Also, the kinetics of the radiotracer after explanting the heart is not clear and it is possible that some cellular 'leakage' of radioiodine occurred post-mortem. In addition, the % ID of ^{123}I as calculated by the software was not as accurately predictive of the *in vitro* counts as reported by Carlson et al. in their study using NIS in a mouse model of

subcutaneously implanted tumours. In our opinion, this reflects the low image resolution of the small-animal SPECT-CT system. The cardiac borders are more difficult to map in the abdomen due to the close proximity to the bowel loops. Use of high-resolution clinical grade CT scans to map the regions of interest as well as a translation of this system to the orthotopic heart transplant model is likely to produce images with a sharper definition of heart borders to overcome this problem. The drawbacks associated with the use of CT to define cardiac shadows could likely be overcome with the use of Magnetic resonance imaging (MRI). MRI is being increasingly used in the non-invasive quantification of cardiac function[175], and can be used to generate high-resolution images with a better soft tissue definition. MR is also increasingly used to quantify function and assess wall motion abnormalities in a study that is free of ionizing radiation. In addition, myocardial perfusion in response to pharmacological pressors could be assessed in the same study. However, spatial resolution and sensitivity of currently available imaging techniques lag behind rapid advances made in vector technology.

In addition, quantification of images obtained at a definite time point was based on the assumption that radioiodine uptake by all NIS expressing cells is uniform in time. This assumption was essential due to the lack of data related to the intracellular kinetics of the radiotracer. Preliminary evidence indicates a rapid efflux of the radio probe from the cell. The requirements of cardiac gene therapy are slightly different from those in gene therapy for the treatment of cancer. In cancer gene therapy where expression of hNIS offers the potential to follow up gene transfer with a therapeutic dose of radioisotope, the ^{131}I must be trapped

effectively within the cells for a definite period to allow for a cytolytic effect. Although there is an initial efficient uptake this is followed by a rapid efflux within 20m, with less than 0.5% of the injected ^{131}I retained by the tumour by 24 hours. [132, 176] Attempts to ensure retention of the radioisotope to produce a cytolytic effect have included the use of Lithium[177], concurrent thyroid peroxidase (TPO) gene transfer [178, 179], administration of low iodine diets and thyroid ablation . These strategies ensure a high circulating level of TSH and therefore a higher NIS expression. However, in models of cardiovascular disease where hNIS is used purely as a marker of the presence of a second therapeutic gene, it is desirable to avoid organification of the iodine and allow for a rapid efflux of the isotope out of the cell to avoid a cytotoxic effect.

5.2 Surgical Limitations of the model

Conditions of cardiac perfusion mandatory to cardiac preservation do not always support efficient gene transduction. In addition, the heterotopic heart transplant model used in this work does not reflect the loading conditions faced by the heart in the life-supporting orthotopic position. Although the myocardium is optimally perfused, the left atrium and ventricle are only partially loaded and both ventricles face an uncertain afterload due to end-to side anastomoses to abdominal vessels. For this reason, it would be inappropriate to carry out studies involving genes affecting haemodynamics in this model. These questions could be better addressed in primate models of orthotopic cardiac transplantation. The use of *ex vivo* perfusion technique is feasible in the large animal model[180]. This method was not used in this study due to constraints with the production of large

quantities of the vector. However, the translation of this technique to humans would be straightforward due to the expertise already gained with SPECT imaging of the heart in clinical studies. The use of SPECT/CT in the transplanted human heart would also facilitate precise quantification of cardiac dimensions and exact sites of gene transfer. Imaging of the heterotopic heart also differs from clinical conditions due to the soft tissue attenuation due to surrounding bowel loops. In the thorax, natural contrast due to the presence of air in the lungs would make easier to visualize the cardiac margins.

5.3 Directions for Future Research

The principle of non-invasive monitoring of transgene expression could be extended to the use of soluble marker peptides to monitor transgene expression. Bicistronic adenoviral vectors coding for hNIS and soluble extracellular domain like human carcinoembryonic antigen (hCEA) and human chorionic gonadotropin (hCG) could be used to monitor gene expression. Non-invasive imaging of radioiodine uptake and serum quantification of soluble markers could be performed with the use of computed tomography (CT) co-registered with micro-SPECT [167] following adenoviral gene transfer. These experiments have been commenced in the lab and initial results look promising.

Some of the drawbacks associated with a potent immune response to the adenoviral vector could be overcome with the use of newer ‘gutted’ adenoviruses which are devoid of all viral coding sequences [181, 182]. Other potential solutions include the use of different serotypes of adenoviruses, non-Ad viral vectors

(Retroviral, Lentiviral, adeno-associated viral vectors) or non-viral vehicles such as endothelial progenitor cells (EPC).

The promise of newer vectors like Adeno-associated virus (AAV) that track to the myocardium with little promiscuous infection of other organs makes intravenous administration of genes a possibility[183]. Evidence suggests that Adeno-associated virus (AAV) vectors induce the pro-inflammatory cytokine response to a lesser degree compared to adenoviral vectors resulting in more efficient gene transduction[184]. Further understanding of the antigenic determinants and mechanisms of the immune response against vectors and gene products can achieve persistent transgene expression. Recently we have made AAV vectors coding for NIS, that unlike adenovirus vectors they do not encode viral proteins; in this way the transduced cells are not immunogenic. Preliminary *in vivo* data are looking promising.

Cell-based therapy is dependent upon the delivery of cells derived from the bone marrow, peripheral blood, adult heart, adipose tissue or the human embryo to the region of interest [185, 186]. Attention has been focused on the potential ability of these cells to repair or regenerate areas of ischemia in the myocardium [187-189]. Bone marrow derived haematopoietic and mesenchymal stem cells, cardiac stem cells, adipose tissue derived stem cells and embryonic stem cells have all been considered for the repair of infarcted myocardium as well as to limit the damage caused by ongoing ischaemia [185]. The advances made in the field of cell-based therapy for cardiovascular disease open up the possibility of using stem cells as delivery vehicles for therapeutic genes. The autologous nature of stem cells offers

the potential advantage of a muted immune response to the courier. In addition to this application, stem cells could be used as delivery vehicles for exogenous therapeutic genes in models of heart disease. Real-time imaging of these cells by nuclear imaging techniques could lead to a better understanding of the kinetics of these cells within the heart. This could be accomplished in several ways, viz the use of radiolabelled cells tracked by PET imaging or the use of reporter genes followed by the injection of a radioligand imaged with PET or SPECT. Non-invasive imaging addresses problems associated with tracking of delivered cells [190], washout of cells to other organs [191] and an understanding of the molecular mechanisms underlying the benefits of cell-based therapy. *In vivo* tracking of cells is essential to validate functional results[192] and this process could be better monitored and followed by the use of reporter genes that permit non-invasive imaging[185].

With the use of with the use of a NIS reporter system the anatomical distribution of an associated therapeutic gene as well as its transduction kinetics in the transplanted heart can be determined with relative ease. The success of this model in visualizing gene transfer justifies development of models to image gene transduction in transplanted hearts with disease. Eventually, genes could be used in a therapeutic role to affect disease states in cardiac transplantation perhaps in concert with other allograft-protective pharmacological therapy.

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7.1 APPENDIX A

Micro-SPECT/CT Image Acquisition Protocol

(Protocol formulated by Dr S Carlson MD[167])

All animals were imaged under sedation. 1000 μCi of ^{123}I was injected into the tail vein and micro-SPECT/CT imaging commenced after 20 minutes. Syringe activity was measured prior to and post-injection to quantify the injected dose accurately. Micro-SPECT images were obtained under a high-resolution gamma camera (X-SPECT, Gamma Medica®-Ideas Inc., CA) with a low energy high-resolution parallel hole collimator with 64 projections at a rate of 10s per projection and an acquisition time of 13m 46s. CT images were obtained under a circular orbit at a thickness of 50μM per slice under the same scanner without moving the animal in between. This ensured accurate co-registration of the axial CT images with the micro-SPECT maps.

Gamma-Medica® XSPECT system information:

SPECT scans:
Collimator: low energy, high-resolution parallel-hole
Field of view: 12.5 cm
Acquisition time: 13:46 min
Projections: 64
Time/projection: 10s
Reported resolution: 1-2mm

CT scans:
Orbit: circular
Slice thickness: 50μm
Images: 256
Acquisition time: 1minute
Voltage: 80kVp; Current: 0.25mA
Reported resolution: 43μm

7.2 APPENDIX B

Protocol for calculation of explanted heart ^{123}I uptake by analysis of SPECT/CT images

(Protocol formulated by Dr S Carlson MD[167])

Calculation of Heart ^{123}I Uptake Using Micro-SPECT/CT (XSPECT) and Image Analysis Software:

1. **Dose Calibrator Measurements:**

   - For ^{123}I: Dial setting 277
   - Set explanted heart (tissue) in chamber
   - Final correction factor:
     - True Measured Tissue Activity = (x) (1.11) (0.76);
       (where (x) = radio-isotope uptake measured by DC)

2. **Conversion Equations Based on Imaging of ^{123}I Standard:**

   - **Planar:**

   $$\text{Tissue activity X } (\mu\text{Ci}) = \frac{(47.93\ \mu\text{Ci})\ (\text{measured tumor ROI counts})}{(25{,}797\ \text{counts})}$$

   - **SPECT (or SPECT/CT):**

   $$\text{Tissue activity X } (\mu\text{Ci}) = \frac{(47.93\ \mu\text{Ci})\ (\text{measured tumor ROI counts})}{(1.10 \times 10^{7}\ \text{counts})}$$

3. **Activity Conversion Factors:**

   a. **Planar:**
      i. ROI Analysis = 25,797 counts

ii. Conversion factor: 1.86 x 10^{-3} µCi per measured count

b. <u>SPECT (or SPECT/CT):</u>

i. Region of interest (ROI) Analysis = 1.10 x 10^{7} counts

ii. Conversion factor: 0.044 x 10^{-3} µCi per measured count

4. <u>Isotope Decay equation:</u>

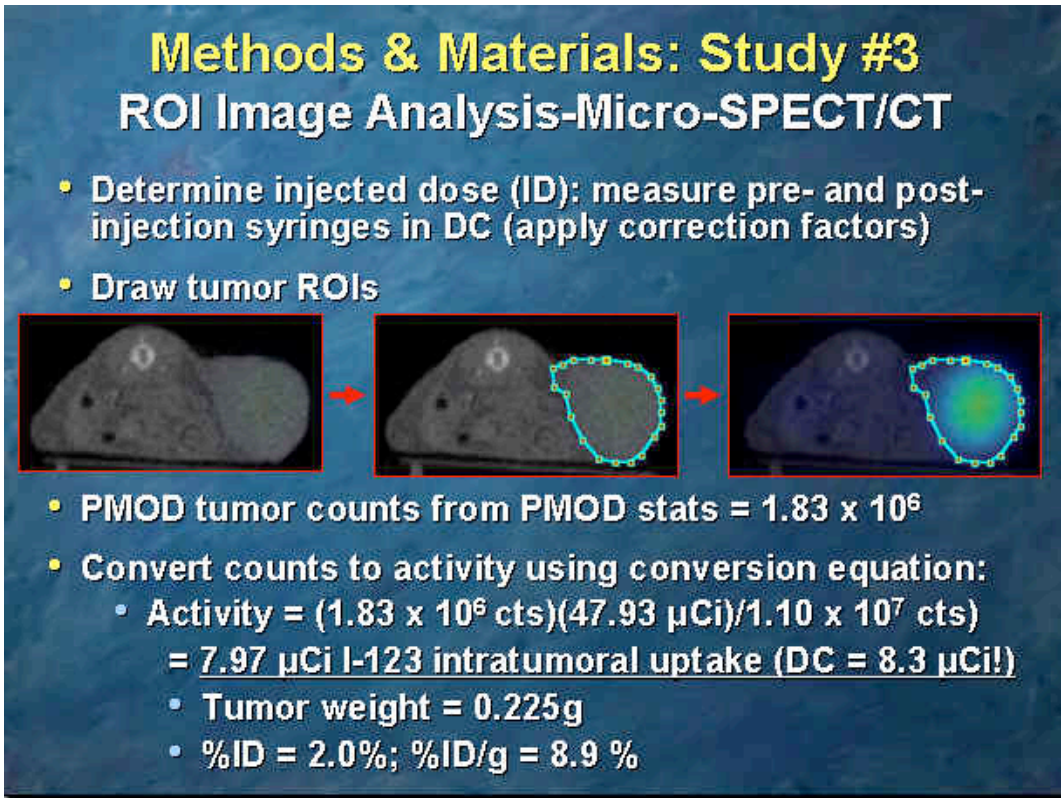
a. Activity (A) = $A_o e^{-\lambda t}$

i. λ = decay constant = 0.693/ $t_{1/2}$

ii. t = time elapsed (hrs)

$A = A_o e^{-(0.693t/t1/2)}$

<u>Summary of Region of Interest (ROI) analysis:</u>



***Use the calculated tissue activity/known injected dose to obtain %ID in the tissue and %ID/g!**

***ROI analysis is the same for planar imaging, but use a different conversion equation:**

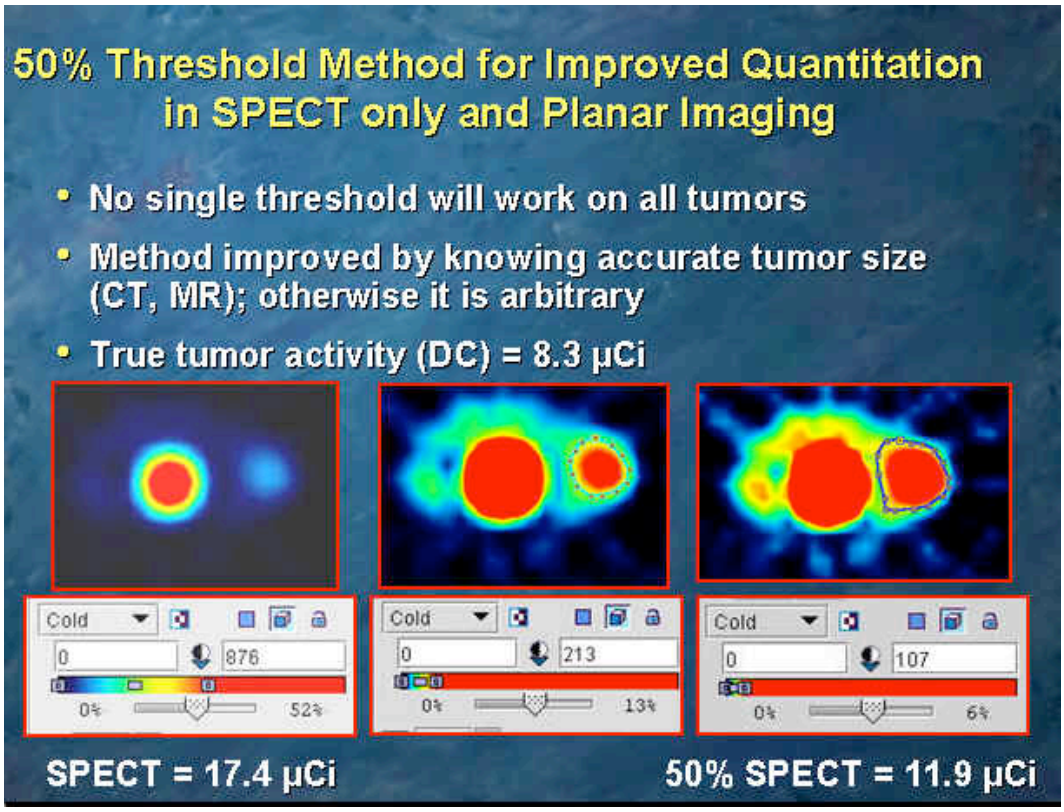
- Activity X = (47.93 microCi)(PMOD tumor counts planar image)/25,797 counts

***Tissue Volume Conversion Factor (it does not give you true volume on PMOD stats analysis):**

- Conversion factor: Vol_{PMOD} (cc) x ($0.31mm^3$)

Example: → Vol_{PMOD} = (14.3cc) x ($0.029791mm^3$) = 0.43ccm = $4.3mm^3$

Summary of 50% Threshold Analysis for SPECT Only Images:



50% Threshold Method: Quantitation of SPECT Images: Step-by-Step

1. Increase image intensity display until you think the yellow or red area is as large as the size of the tissue (need to pick either yellow or red color to use as your reference for the entire analysis—either is okay, but be consistent!)

2. Go through each SPECT image that the tissue is on and place an ROI around the yellow or red area (whichever one you picked)

3. Look at Stats analysis on PMOD and determine maximum pixel count

4. Multiply the number obtained in (3) by 50%

5. Use this new calculated number and put it in the right box of your image intensity bar (leave the left box zero)

6. Go through the entire tumor again on all SPECT images in which it is visualized outlining the yellow or red area (whichever you used before) and determine new total tissue counts

7. Convert to activity with the usual activity conversion equation described earlier

7.3. APPENDIX C

Publications

- **Rao VP,** Miyagi N, Ricci D, Carlson SK, Morris JC, Federspiel MJ, Bailey KR, Russell SJ, McGregor CGA:
  Sodium iodide symporter (hNIS) permits molecular imaging of gene transduction in cardiac transplantation.
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