GENETIC MANIPULATION OF THE TRANSPLANTED HEART BY ADENOVIRAL-MEDIATED GENE TRANSFER

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Declarations

I hereby declare that this thesis and the work described in it has not been submitted in candidature for any other degree and except where otherwise stated, is the product of my own investigation. This thesis is the result of experimental work carried out during the period of 1/9/95 to 31/8/96 at Mayo Clinic and Foundation, Rochester, Minnesota, U.S.A.

Date: 1st August, 1998

(John Yin Ming Yap)

Abstract

Heart transplantation is currently a viable option for the treatment of patients with end-stage cardiac disease. Although improved survival has been achieved over the last decade, transplant recipients remained vulnerable to the risks of rejection, infection, the effects of immunosuppression and accelerated transplant atherosclerosis.

Recent advances in molecular biology and recombinant DNA technology have fostered the concept of gene transfer. Successful gene transfer is dependant on the properties of vectors used in delivering genes to the target cells. The vectors used to date include adenovirus, retrovirus, adeno-associated virus and plasmid-liposome. Replication defective adenoviral vectors are currently the agents of choice for *in vivo* cardiovascular gene transfer because of their relative efficiency and ability in transducing non-dividing cells. Disadvantages of adenoviral vectors include transient duration of transgene expression and potential cytotoxicity.

Gene transfer to the transplanted heart may represent a novel approach to the study of transplantation biology and may have therapeutic potential in the management of human transplantation disease. The availability of the donor heart at the time of harvest and the period of cold preservation may allow for a prolonged viral vector dwell time which would not be possible in *in vivo* vascular wall gene transfer. This prolonged dwell time may lead to

enhanced gene transfer in this setting. Also, the necessary use of immunosuppressive agents in transplant setting may modulate the potential cytotoxicity and duration of transgene expression of adenoviral-mediated gene transfer. In the cardiovascular system, nitric oxide (NO) derived from the vascular endothelium regulates vascular tone. Its other properties include inhibition of proliferation of smooth muscle cells, adhesion and activation of leukocytes and platelets. In addition, reduced bioactivity of nitric oxide is a feature of atherosclerosis and vascular injury. Therefore, overexpression of nitric oxide may be beneficial in modifying accelerated transplant atherosclerosis.

The focus of this thesis was on adenoviral-mediated gene transfer in the heart transplant setting. It addressed the feasibility and favourable conditions to achieve efficient gene transfer in the donor heart during cold preservation. In particular, the effect of increased coronary distension during viral vector dwell and the potential role of warm ischaemia, during the transplant procedure, on efficiency of gene transfer were examined. Efficiency of gene transfer was assessed by histochemical staining of the reporter gene encoding for β-galactosidase. Further evaluation of the effect of temperature on gene transfer by *in vitro* study was performed. The major limitation of adenoviral vectors, transient duration of transgene expression, was examined. The effect of immunosuppression on duration of transgene expression was assessed in both syngeneic and allogeneic transplantations. Transgene expression in transplanted hearts were assessed at 1, 4, 8 and 12 weeks. Finally, the

endothelial nitric oxide synthase gene was transferred into the donor hearts and the potential functional consequences studied.

The current study confirmed the feasibility of adenoviral-mediated gene transfer to the transplanted hearts. However, the efficiency of gene transfer was markedly affected by the conditions of gene delivery. The study in Chapter 3 demonstrated that coronary distension during the period of viral infusion could significantly improve gene transfer. This was further enhanced by allowing virus dwell during the period of warm ischaemia when transplantation was performed. Interestingly, virus dwell during the period of warm ischaemia alone did not improve gene transfer efficiency even though it was noted that gene transfer was somewhat more accentuated in subepicardial areas of organising ischaemia where rewarming occurs quicker. However, *in vitro* study of endothelial cells showed that gene transfer at 37° C was significantly better than at 4°C or 10°C. Another major limitation of the adenoviral vector is the transient nature of transgene expression. This may pose a potential limitation to its effectiveness for clinical gene therapy. The need for immunosuppression in transplantation may modify this immune response and facilitate prolonged transgene expression. The study in Chapter 5 showed that transgene expression persisted significantly longer in both the immunosuppressed syngeneic and allogeneic animals compared to the non-immunosuppressed animals. Therefore, transgene expression using adenoviral vectors is prolonged by immunosuppression in the heart transplantation setting. The concluding study confirmed that endothelial nitric oxide synthase

gene, a biologically active and relevant gene in the cardiovascular setting, can be successfully transferred into transplanted hearts by the methods defined earlier. However, overexpression of the recombinant protein in this model did not did not result in significant changes in functional myocardial activity and coronary perfusion. Whether the overexpression of eNOS may modify accelerated transplant atherosclerosis remains to be determined.

These findings may have important implications for the future development of adenoviral-mediated gene transfer in the heart transplant setting.

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Abbreviations

AdeNOS	Adenoviral vector encoding for bovine endothelial nitric oxide synthase gene
AdLacZ	Adenoviral vector encoding for β-galactosidase gene
CAR	Coxsackie and Adenovirus Receptor
cDNA	Complimentary DNA
eNOS	Endothelial constituitive nitric oxide synthase
iNOS	Inducible nitric oxide synthase
HUVEC	Humna umbilical vein endothelial cell
L-NMMA	N^G-Monomethyl-L-Arginine
MOI	Multiplicity of Infection; equivalent to number of viral particles per cell
NOS	Nitric oxide synthase
pfu/ml	Plaque forming unit per ml
X Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

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Chapter 1 : Introduction

Brief History of Heart Transplantation

In 1905, Carrel and Guthrie reported on a series of heterotopic heart and lung transplantations in dogs during the course of establishing the techniques for vascular surgery. This work was taken further by Mann and workers in the 1930s. In the mid-1940s in Russia, Demikhov performed a series of experiments showing the technical feasibility of intrathoracic heterotopic heart transplant and its ability to sustain circulation. However, it was not until the advent of cardiopulmonary bypass, when in 1960 saw the publication of Lower and Shumway's classic paper[1] in orthotopic cardiac transplantation, integrating advances in surgical techniques, recipient support, and donor heart preservation into a single approach. This established the basis for human heart transplantation.

In the field of early immunology, in the 1940s, the work of Medawar on skin grafts revolutionised the understanding of transplantation and the development of the concepts of immunology. Progress in laboratory research was translated into clinical reality when the first successful human orthotopic cardiac transplantation was performed by Barnard in 1967 at Groote Schuur Hospital. This led to an increase in the number of cardiac units world-wide undertaking this procedure. However, early results of heart transplantation were generally disappointing, and the procedure was largely abandoned by 1970 with the notable exception of Stanford under the direction of Shumway.

Through the 70's and the 80s, progress achieved in laboratory and clinical research especially at Stanford resulted in improvement in the survival of heart transplant patients. Significant milestones contributing to the success include the use of surveillance endomyocardial biopsy[2], improvement in immunosuppressive agents especially cyclosporin A, and the diagnosis and management of rejection. Other important factors include advances in recipient and donor selections and the development of distant heart procurement programmes.

Challenges in Heart Transplantation

Heart transplantation is now a viable option for the treatment of end stage cardiac disease. Transplant recipients have approximately 80% survival at 1 year, with survival extending to 60% at 5 years[3]. Although improved survival has been achieved over the last decade, transplant recipients remained vulnerable to the risks of rejection[4,5], infection and the effects of immunosuppression. In addition, long term survival is severely compromised by the condition of accelerated transplant atherosclerosis[6]. Further constraint to the widespread application of cardiac transplantation is the availability of donor organs.

Rejection and Immunosuppression

Cardiac allograft rejection is a result of a complex immunological process involving the recognition of the allogeneic histocompatibility antigens of the grafted tissue by the host immune system. The most important histocompatibility antigens are those of the major histocompatibility complex (MHC), known in human as the HLA system. This distinguishes the "self" from the "non-self" and there is a high degree of polymorphism within the system. In addition, numerous other antigens of the minor incompatibility groups also contribute to the rejection process. The three main components of allograft rejection include donor specific T cytotoxic lymphocytes, non-specific imflammatory cells and anti-donor antibodies. The main strategy for preventing and treating transplant organ rejection in clinical practice is the use of immunosuppressive agents. Other potential strategies for overcoming rejection are MHC-matching and the induction of tolerance. The application of MHC-matching is hampered by the high level of polymorphism in the genes encoding for HLA antigens and thus, the likelihood of a perfect match is remote. Also in clinical practice, this is further restricted by the ischaemic time constraint and the shortage of available donor organs. Transplantation tolerance refers to the elimination of the immune response to the antigens of the allograft while retaining the immune response to all other antigens in the environment. Presently, the induction of transplantation tolerance remains experimental in cardiac transplantation.

All transplant recipients require indefinite immunosuppression to prevent rejection. Current immunosuppressive regimens are for most part immunologically non-specific in their mode of actions. Despite major improvements in immunosuppressive therapy, allograft rejection remains an important cause of morbidity and mortality in cardiac transplant recipients. New immunosuppressive strategy that specifically targets the transplanted heart may avoid the side effects seen in present immunosuppressive regimens. Gene transfer technology may potentially offer such a strategy.

Accelerated Transplant Atherosclerosis

Accelerated Transplant Atherosclerosis has emerged as the major cause of morbidity and mortality in long-term heart transplant recipients[7,8]. The prevalence of this condition was found to be 3% at 1 year and 40% at 5 years in an angiographic study[7]. However, intravascular ultrasound imaging, a more sensitive method for detecting and quantitating intimal vascular wall thickening, highlighted the underestimation of the prevalence of this condition angiographically[9,10]. The predominant pathological findings are diffuse, concentric intimal thickening and perivascular inflammation involving large and medium-sized epicardial arteries to arterioles. The vascular lesion is characterised by marked proliferation of intimal smooth muscle cells with lipid accumulation and mononuclear cell infiltration. It differs from "native" coronary atherosclerosis in its generalised distribution throughout the coronary

vasculature and its rapid progression. Despite these differences, there are morphological and functional similarities between accelerated transplant atherosclerosis and "native" atherosclerosis.

The pathogenesis of this condition remains the subject of intense debate. It is most likely to be multifactorial involving both immune and non-immune mechanisms. The common pathway to the development of this condition may be the "response to injury"[11,12]. In heart transplantation, the causes of endothelial injury include cold storage ischaemic injury, ischaemic-reperfusion injury, immune injury from acute and chronic rejections and viral infections[13]. The biology of accelerated transplant atherosclerosis is still poorly understood and only considerable progress in its understanding may eventually lead to strategy in the treatment and prevention of this devastating condition. Currently, the only effective treatment is retransplantation.

Gene Transfer and Therapy

Since 1953 when Watson and Crick elucidated the structure of DNA, advances in molecular biology and recombinant DNA technology have cumulated in the potential use of gene transfer for the treatment of genetic and acquired diseases. Gene transfer is the introduction of recombinant DNA into the target cell. This can be divided into germ cell or somatic gene transfer.

Early gene therapy concept involves the transfer, substitution or deletion of genes at germ cell level. The potential danger of mutagenesis and unpredictable results of germ cell manipulation with its ethical and biological consequences resulted in decreased interest of this technique for gene therapy. The main potential application of germ cell gene transfer is in the development of transgenic animals in the field of xenotransplantation. The main focus for gene transfer at present is into somatic cells. The ability to introduce therapeutic genes into target cells resulting in the expression of the genes and the production of the therapeutic proteins may have many beneficial clinical applications.

Somatic gene transfer can be carried out *ex vivo* or *in vivo*. In the former, the recombinant gene is transferred to the target cells in the laboratory after which they are returned into the host animal. In contrast, *in vivo* procedure involves the transfer of the recombinant gene into the host animal directly. Both techniques have applicability and the choice of the strategy depends on the target cells and the condition to be treated. Most of the human protocols on gene transfer to date used the *ex vivo* technique. The type of diseases and potential target cells or systems for gene therapy are diverse [14]. Successful gene therapy requires an efficient method of gene delivery that targets relevant cells and results in stable gene expression to achieve therapeutic outcome without unnecessary deleterious side effects.

Vector Systems

Early experiments with viruses and naked DNA complexed with calcium phosphate established the techniques for *in vitro* gene manipulation and genetic material transfer. The basis for gene therapy was then contemplated [15]. In the 1980s, the development of replication defective retroviral vectors and suitable packaging cell lines helped progress toward gene transfer experiments. The genetic transfer process is usually aided by the use of a vector system to deliver the recombinant gene into the intracellular site of the target cell for it to function appropriately. From an early stage, it was realised that successful gene therapy is dependant on efficient methods of gene delivery. The vector systems to date can be broadly divided into non-viral and viral vector systems (Figure 1.1). Non-viral vectors include naked DNA, liposome and methods incorporating receptor-mediated endocytosis. Viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, HIV/Lentivirus and Sendai virus/HJV.

The ideal vector system for gene transfer should: a) have no insert DNA size constraint, b) target either entry into specific cells or limits expression to target cells, c) not evoke an immune response, safe for the recipient and environment, d) be stable and free from insertional mutagenesis, e) be easily produced and in high titre/concentration and f) be possible to regulate the level of transgene expression. At present no such vector system exists. Currently, cationic liposomes and viral vectors are the most commonly

used agents in the field of cardiovascular gene transfer. The advantages and disadvantages of these vectors will be considered.

Non-viral Vectors

Liposome-Mediated Gene Transfer

Liposomes are self-assembling colloidal particles in which a lipid bilayer encapsulates a fraction of the surrounding aqueous medium. Cationic liposomes are positively charged lipid vesicles that incorporate negative charged recombinant DNA and facilitate the delivery of the DNA into the target cells (cell membrane being negatively charged) through fusion with the cell membrane or receptor-mediated endocytosis. Delivery of liposomes is non-specific as there are no specific receptors involved in binding and entry of these complexes into target cells. In addition to vascular wall gene transfer[16,17], cationic liposomes have been used to deliver genes *in vivo* to lung epithelial cells [18], porcine arteries [19] and brain tissue and the systemic circulation. The principal disadvantages of liposome-mediated gene transfer are poor transduction efficiency and transient gene expression. Potential advantages of this system include the fact that liposomal-mediated DNA remains episomal in the nucleus, thus avoiding problems of random insertional mutagenesis. Liposomal vectors, with no viral sequences, have lower immunogenicity and there is no cDNA size constraint in vector construction. Liposomal vectors have been shown to have very good biosafety profile [20,21]. With

improvements in formulation resulting in greater efficiency of gene transfer, this vector system holds great potential for the future [22].

Receptor-Mediated Endocytosis

Gene transfer is achieved by conjugating the recombinant DNA with a cell-specific carrier molecule which acts as a ligand for the target cell receptor [23]. The advantage of this method is the potential for targeting gene transfer to specific receptors expressed by the cells of interest. The hepatocyte-specific asialoglycoprotein receptor has been targeted by an electrostatic complex, asialorosmucoid-polylysine conjugated to the DNA [23] of interest in gene transfer to the liver. Another example is the transferrin-polylysine/DNA conjugate which binds to transferrin receptors [24]. The efficiency of this system is enhanced by the addition of substances, such as chloroquine, which disrupt the endosome/lysosome pathway facilitating the delivery of DNA into the nucleus. Simultaneous addition of replication defective adenoviral vectors is another method to disrupt the endosome/lysosome pathway and increase the efficiency of gene transfer with this method.

Viral Vectors

Retrovirus

Retroviruses were the first viral vectors used in human gene therapy [25]. Retroviruses are RNA viruses which have the capacity to convert RNA to DNA by a viral enzyme, reverse transcriptase. It comprises an inner core which include the RNA, virally coded protease, reverse transcriptase and integrase enzymes enclosed by a phospholipid envelope. The virus infects cells by specific binding of an envelope protein to a cell surface receptor. The RNA is then transferred to the nucleus and during this process RNA is converted to double stranded DNA by reverse transcriptase. The double stranded DNA is integrated into the host cell chromosome, a process mediated by the enzyme integrase [26]. The integrated viral DNA is termed the provirus and functions like an independent gene. The integration is a semi-random process with multiple sites used although some are used more frequently than others [27]. RNA is transcribed from the integrated provirus and translated to make viral proteins. These proteins combine with full length RNA to form new viral particles.

The most commonly used retrovirus in gene transfer experiments is the Moloney murine leukaemia virus. Retroviral-mediated gene transfer has been used successfully in the *ex vivo* setting [28] which involves the removal of

relevant target cells from the host and transfecting them *in vitro* before returning the modified cells back to the host.

However, the major limitation of the retroviral vector for *in vivo* gene transfer is its inability to transduce non-replicative cells. Replication of target cells is required for proviral integration [29]. Cells of the arterial wall and cardiomyocytes are usually quiescent and therefore gene transfer using retroviral vectors is inefficient. As retroviral RNA is stably integrated into the host cell chromosome, random insertional mutagenesis is a concern. Other disadvantages include the inability to grow the virus to high titre, instability from handling and rapid inactivation in primate serum [30]. This may limit its use in large animals or human applications. Direct *in vivo* gene transfer into vascular cells with retroviral vectors has also been attempted with transduction efficiencies even lower than those found *in vitro* [31]. However, a major advantage of retrovirus-mediated gene transfer is prolonged transgene expression as a result of integration of the provirus into the host cell chromosome.

Interestingly, a lentiviral vector system based on human immunodeficiency virus had been developed which successfully transduced non-dividing cells [32]. Lentiviruses, unlike most retroviruses, can transduce non-replicative cells and the transferred genes are integrated into the host cell chromosome which is a desirable feature for long-term gene expression. Therefore, this retroviral vector overcomes the primary disadvantage of other retroviral vectors which is the inability to transduce quiescent cells while

retaining the integration benefit. However, currently there are considerable biosafety implications from using lentivirus vectors which may restrict their use in human gene therapy.

Adeno-associated Virus (AAV)

Adeno-associated virus is a member of the parvovirus family. The AAV genome is a single-stranded linear DNA molecule with terminal repeats at both ends which fold into hairpin structures and function as origins of replication [33]. AAV depends on the co-infection of a helper-virus, either an adenovirus or herpesvirus for efficient replication. One potential advantage of AAV is that it preferentially integrates into chromosome 19. Other important features are a) its ability to infect a wide variety of mammalian cells, b) ability to transduce non-dividing cells, c) stable and replicates to high titre. Disadvantages of AAV include the absence of a system of growing high titre AAV stock. Secondly, the procedure for growing AAV recombinants is cumbersome. The growth of a recombinant stock requires the presence of both AAV and helper virus genes and therefore the resulting recombinant is contaminated by wild type adenovirus and sometimes wild type AAV particles. Further modifications of this vector system may overcome these disadvantages and provide a promising vector for future gene therapy.

Sendai virus-DNA-Liposome Complexes

In this method, inactivated Sendai virus (hemagglutinating virus of Japan), and the nuclear protein, high-mobility group 1 (HMG1) are complexed with liposomal DNA to achieve efficient gene transfer. The HVJ facilitates membrane fusion at the cell surface at neutral pH and facilitates the introduction of DNA directly into the cell cytoplasm, avoiding lysosomal degradation [34]. The HMG1 nuclear protein increases the efficiency of nuclear translocation and expression of the DNA [35]. This system has been used successfully to introduce and express the human insulin gene [36] and the human renin gene in adult rat liver [37]. This system has also been used to transfer genes to the rat carotid artery *in vivo* [38] and the myocardium [39] efficiently. Limitations to this system include transient nature of gene expression and the technical expertise required in the preparation of the complex prior to transduction. In addition, the experiences on this vector system have been restricted to a few laboratories.

Adenovirus Vectors

Adenoviruses are intermediate-sized DNA viruses with genomes consisting of linear double-stranded DNA molecules of 32,000 to 36,000 base-pairs. The virion is about 70nm in diameter, consisting exclusively of protein and DNA. The DNA is contained within an icosahedral protein coat or capsid. Protruding from each of the twelve vertices of the capsid is a fibre

protein which plays an important role in the successful transfection of target cells.

The genome of the adenovirus is divided into two major non-contiguous overlapping regions, early and late, based on the time of transcription after infection. The distinct early regions are E1a, E1b, E2a, E2b, E3 and E4. There is also a late region under the control of the major late promoter. In general, the early genes are involved in the regulation of viral transcription, transformation and DNA replication while the late genes code for viral structural proteins. The adenoviral vectors used to date have the E1 region deleted to prevent them from replicating in target cells (Figure 1.2). However, these vectors could be propagated in the laboratory in 293 cells (a cell line that is transformed by adenovirus 5 DNA and expresses the E1 genome thereby providing proteins in trans) [40]. The successful transduction of target cells by adenoviral vectors requires three distinct, sequential steps: 1) attachment of the virus to specific receptors on the surface of the target cell; 2) internalisation of the virus; and 3) transfer of viral genome to the nucleus where it can be expressed (Figure 1.3).

Replication defective adenovirus vectors are currently the agents of choice for *in vivo* cardiovascular gene transfer [41]. More efficient gene transfer is observed using these agents than any other to date. A major advantage of adenovirus vectors is the ability to transduce non-replicative cells [42]. They can be grown to high titre and are not incorporated into the host cell genome but remain episomal. Random insertional mutagenesis is therefore not a major

concern. However, as chromosomal integration does not occur, the duration of expression of the transgene is limited. Furthermore, systemic administration of adenovirus cannot be repeated due to the presence of host immune responses. Disadvantages of adenoviral mediated gene transfer therefore include potential cytotoxicity, limited duration of gene expression, and the inability to administer a second dose due to immune responses [43].

Despite the drawbacks outlined above, adenovirus vectors have had many applications to date. They have been used to transfer the cystic fibrosis transmembrane conductance regulator gene to the bronchial epithelium of cottontail rats [44], the nasal epithelia of patients with cystic fibrosis [45], and the biliary tract of rats. These vectors have also been used to transfer the gene for the low density lipoprotein receptor to both normal and low density lipoprotein receptor knockout mice and Watanabe heritable hyperlipidemic rabbits [46]. Adenoviral vectors have also been used to transfer genes to skeletal muscle, ventricular myocytes, human blood monocyte derived macrophages, mouse and human hepatocytes, rabbit synovium, and neurones and glia in the brain [42]. Recently, adenoviral vectors have been used to transduce vascular wall cells[47,48]. Adenoviral-mediated transfer of α1-antitrypsin cDNA to human endothelial cells *in vitro* was described by Lemarchand et al [49]. Adenoviral vectors have also been used to transfer the genes for β-galactosidase and CFTR to the carotid artery and jugular vein of sheep *in vivo*. In addition, adenoviral vectors have been used to transfer genes into the heart [50,51].

Gene Transfer and the Heart

The expression of recombinant genes in the human coronary system and myocardium holds promise for the treatment of a number of inherited and acquired cardiovascular diseases.

Wolff et al [52] first described the feasibility of *in vivo* gene transfer to muscle cells by direct injection of naked DNA plasmid. In this series of experiments although gene transfer was successful, the efficiency was extremely low at about 1% of all cells. This raised the question of whether cardiac cells could be similarly transduced. This was addressed by Lin [53] and Acsadi [54], who in separate experiments, reported successful gene transfer into cardiac cells by direct *in vivo* injection of naked plasmid DNA. Again the level of transgene expression was low and successful gene transfer was restricted mainly to the site of injection. Although multiple injections into the heart is possible, the level of transgene expression may still be limited and the potential for myocardial damage resulting from such a manoeuvre would be undesirable. Despite the low level of gene transfer by this method, this does not exclude the possibility that isolated application might be developed. For example, the direct injection of a plasmid encoding the gene for a potent secreted mitogen such as VEGF[55] (vascular endothelial growth factor), where only a small amount of recombinant protein is required to exert an effect, might

stimulate collateral formation in ischaemic areas of the myocardium. Another interesting issue raised by these studies was the duration of transgene expression. Acsadi et al showed the loss of transgene expression in the heart of normal rats after only 28 days. But when these rats were treated with cyclosporine, transgene expression was stable to 60 days. Also, when the injection was done in nude rats, the duration of transgene expression was significantly prolonged. These early observations suggested the role of the immune system in the stability of transgene expression.

Since these early experiments, alternative approaches and vector systems have been developed and used to achieve better efficiency of gene transfer. These include the use of adenoviral, adeno-associated viral, HVJ-liposomal and cationic liposomal vectors. Retroviral vectors had been universally inefficient due to the quiescent nature of endothelial cells and cardiomyocytes. However, Nabel and workers were successful in using this vector in transducing vessels following injury [56]. This was probably related to cellular proliferation following injury to the vessels studied.

Adenoviral vectors have been used increasingly in cardiovascular gene transfer because of their relative efficiency in transducing a wide variety of cells [42]. Stratford-Perricaudet et al [57] utilised a replication-defective recombinant adenovirus encoding for the reporter gene, β-galactosidase, for studying the specificity, efficiency and stability of transgene expression in the heart following intravascular administration of the vector into a neonatal mice. Transgene expression was seen in the liver, intestine, lung, skeletal muscle and

heart. Only approximately 0.2% of cardiac cells were found to stain positive for the reporter gene. However, he found that transgene expression persisted for up to a year albeit in decreasing amount. When the experiment was repeated in adult mice, efficiency was reduced and duration of transgene expression was greatly shortened. Another point highlighted in the study was that ineffectiveness of intravenous injection in targeting the heart for gene transfer. Coronary arteries present a more challenging target for gene transfer compared to peripheral arteries due to access and the critical importance of maintaining distal perfusion. Nabel et al[58] used a double-balloon catheter for site specific gene transfer to peripheral vessels successfully. Access to the coronary arteries for the purpose of gene transfer might be possible with minor modifications to standard catheterisation and angioplasty techniques. Chapman et al[59] used a modified percutaneous perfusion balloon catheter to deliver a reporter gene in liposome into coronary arteries of dogs. The efficiency of gene transfer was hampered by the vector system used. The potential for this technique was supported by French et al [60] who successfully transduced coronary arteries of pigs using a similar catheter. The study demonstrated that adenoviral vector was significantly more effective in achieving gene transfer than liposomal system. Despite this, the absolute level of recombinant protein per target segment was still relatively low and the overall level of gene transfer was low. In contrast, Leiden and colleagues achieved efficient level of gene transfer to the whole heart by infusing a replicative-defective recombinant adenovirus into the coronary arteries of

rabbits using a percutaneous angiographic catheter system[61]. Animals were sacrificed 5 days to 2 months following virus infusion and the myocardium analysed histochemically for transgene expression. Cardiomyocytes, fibroblasts, endothelial cells and smooth muscle cells were successfully transduced. All areas of the heart including atria and ventricles showed gene expression. Efficiency of gene transfer was high. However, the transgene expression had mostly disappeared after 1 month following virus infusion. Another interesting finding was the absence of inflammatory response or myocardial necrosis. This is important because of the question of vector-induced toxicity.

These studies demonstrated the feasibility of gene transfer to the myocardium with an adenoviral vector via the intravascular route. These observations were supported by other studies using adenoviral vectors for gene transfer into the myocardium [51,62,63]. The route of adenoviral vector administration in these studies was mainly by direct injection into the myocardium. Although this was a much more invasive technique, specific area of the myocardium could be targeted. In one study [64], an area of myocardial infarction was targeted by direct injection with the recombinant adenoviral vector. It showed a lower level of gene transfer in the peri-infarcted area compared to normal myocardium. In addition, most of these direct injections into the myocardium using the adenoviral vectors showed inflammatory responses particularly along the needle tract [62,64]. The inflammatory responses

were probably due to both the vectors and the traumatic effect of the needle. This may limit the technique for future gene therapy.

Gene Transfer in the Transplanted Heart

Despite the success of allograft heart transplantation in recent years [3], complications including rejection, toxic effects of immunosuppression and accelerated transplant atherosclerosis continue to be major problems. Gene transfer may offer a new strategy for studying these processes and may offer a potential strategy for adverting them. Heart transplantation may be ideally suited for gene therapy as explanted donor hearts are commonly maintained in preservation solution for up to 6 hours. During this period, the organ would be an ideal substrate for gene therapy. Defined doses of gene therapy can be applied under controlled conditions of temperature, pressure and concentration.

The first published study looking into gene transfer in the transplanted heart was reported by Wang et al [50]. In this study, plasmid DNA encoding reporter genes was injected directly into the myocardium of heterotopically transplanted hearts following reperfusion. The hearts were harvested at different time points for assessment of gene transfer. Gene transfer was demonstrated for up to 60 days. The weakness in this study was the lack of controls and quantification of gene transfer was by luminiscence

measurement of luciferase activity. Therefore, it was difficult to assess the efficiency of gene transfer as luciferase is a very sensitive marker and endogenous luciferase in rat hearts may distort the results. Although β-galactosidase, as a reporter gene, was also used, the distribution of transgene expression was not clarified in this study.

Access to the donor heart from the time of harvest to the time of implantation may provide a unique opportunity for a prolonged exposure of the transducing vector to the heart. Ardehali et al [65] utilised this advantage using a cationic liposomal vector carrying the reporter gene luciferase in transducing donor hearts at the time of harvest. He injected the vector into the heart via three routes a) apex of the heart, b) right atrium and c) the coronary arteries. The vector was not flushed out of the donor hearts before transplantation. The apical injection revealed transgene expression restricted at the injection site. In the right atrium group, transgene expression was detected mainly in the right ventricle. But in the group that had the vector injected into the coronary arteries, transgene expression was present in both ventricles and also the coronary arteries. This study did not indicate the efficiency of gene transfer. Immunostaining for luciferase was not helpful in clearly identifying the cell types transduced. However, this study did support the feasibility of gene transfer to the transplanted heart and indicated that the intracoronary route may be ideal for widespread distribution. Efficiency may be improved with another vector system.

Sawa et al [66] used the HVJ liposome vector carrying either FITC-labelled oligonucleotide or β-galactosidase successfully in transducing donor hearts via the coronary arteries at the time of harvest in a rat model. The dwell time of the vector was about 32 minutes. Transgene expression assessed 3 days later showed all layers of the myocardium transduced with the reporter genes. There was no signs of toxicity. But no quantifiable value was given for the efficiency of gene transfer. The same group reported on a comparative study between HVJ liposome and cationic liposome vectors which showed that the efficiency was seven times higher using the HVJ liposome vector. [39]. In this study, the group also transduced donor hearts with the HVJ liposome vector carrying the manganese-superoxide dismutase gene (SOD) which resulted in better tolerance to ischaemic-reperfusion injury. Fyfe et al [67] was able to transfer the HLA-B7 (MHC class 1) gene into donor hearts using cationic liposome in a mouse syngeneic heart transplantation model. This showed marked evidence of rejection in the HLA-B7 transduced hearts compared to control even though the level of gene transfer was extremely low. However, the importance of this study related to the feasibility of transferring immunologically relevant and important genes in the transplantation setting.

Lee et al [68], using an adenoviral vector carrying the reporter gene, β-galactosidase, studied the duration of transgene expression in a mouse heterotopic heart transplant model. All donor hearts were stored at 4 ^{0} C for 15 minutes after viral infusion into the coronary arteries prior to transplantation. Results were quantified by counting the number of

transduced cells per mid ventricular section stained histochemically. All cell types were transduced but predominantly cardiomyocytes. Transgene expression decreased with time but this was still present at 30 days after gene transfer. No toxicity was reported. Only one concentration of viral solution was used. The effect of higher and lower concentrations of virus was not addressed in this study.

Many of the basic issues of gene transfer using the adenoviral vector in the heart transplant setting remained unanswered. The optimal doses of virus and the conditions favourable to adenoviral-mediated gene transfer in the transplant setting have not been fully addressed.

Adenoviral Vector and the Immune System

One of the major limitation of adenoviral vectors is the transient nature of transgene expression. This has been consistently shown in a number of studies in different animals[47,54,68-70]. However, transgene expression was prolonged in nude and new-born animals suggesting an immunological basis. A fundamental problem with first generation recombinant adenoviral vectors is that deletion of E1 sequences is insufficient to completely ablate expression of other early and late viral genes or to completely prevent replication of viral DNA [43]. The immune response may be directed against the vector, the transgene product or the leaky viral proteins. Another reason for the loss of

gene expression may be due to the episomal nature of the viral DNA in the transduced cells leading to DNA disintegration. In addition, both the cellular and humoral immune response have been implicated in the loss of transgene expression using adenoviral vectors [71]. Also, the humoral response may be responsible for the ineffectiveness of gene transfer following readministration of the adenoviral vector [72,73]. The use of immunosuppressive agents following adenoviral-mediated gene transfer into muscle cells had resulted in prolongation of transgene expression [74]. Due to the toxic side effects of immunosuppressive agents, the routine use of these agents may not be desirable. In clinical heart transplant, immunosuppressive agents are routinely used to prevent and treat allograft rejection. However, little is know of the effects of the necessary use of immunosuppression in the allograft heart transplant setting on transgene expression. This warrants further investigation.

General Aims

1. Validation of animal model of heterotopic heart transplantation.
2. Experiments to define conditions for efficient adenoviral-mediated gene transfer.
3 Experiments to study the duration of transgene expression in the syngeneic and allogeneic heart transplant setting.
4. Experiments to study the feasibility and effects of transferring a biologically relevant gene, the endothelial nitric oxide synthase (eNOS) gene, in the heart transplant setting.

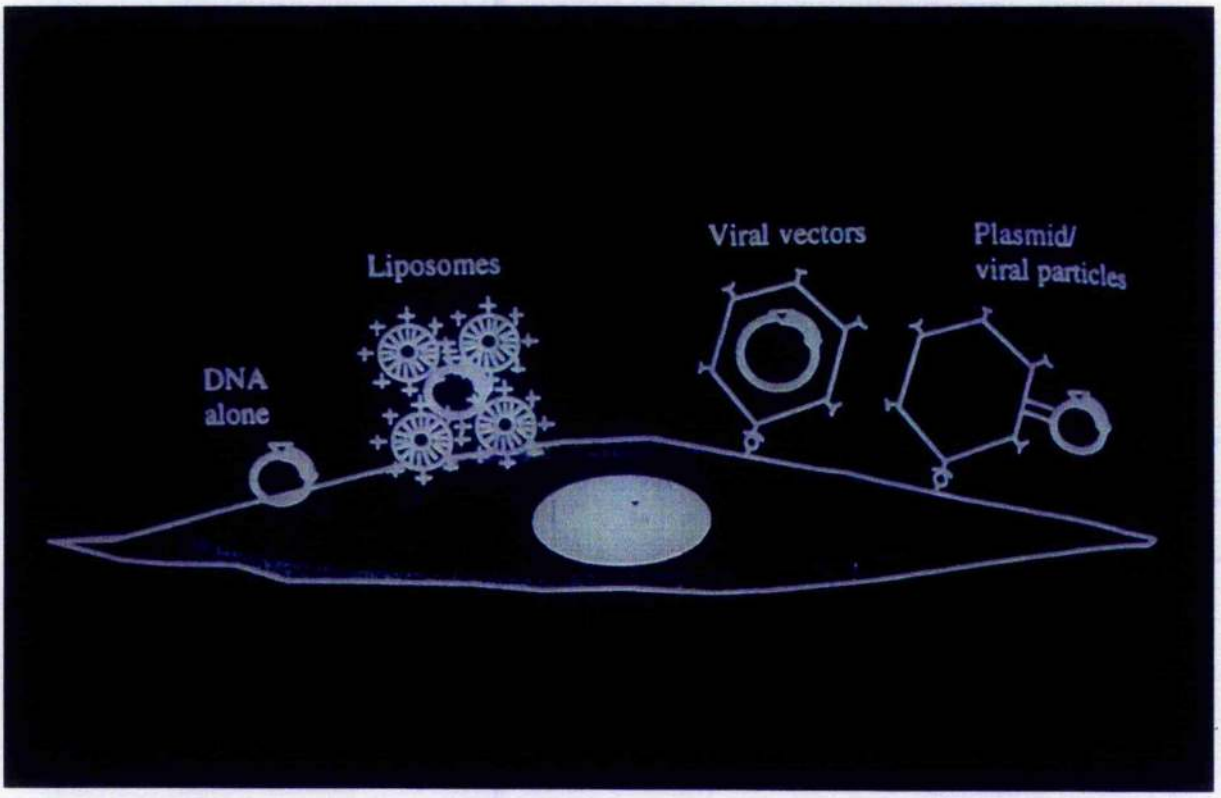

Figure 1.1: Gene transfer vectors

Diagram depicting non-viral and viral vectors used in gene transfer

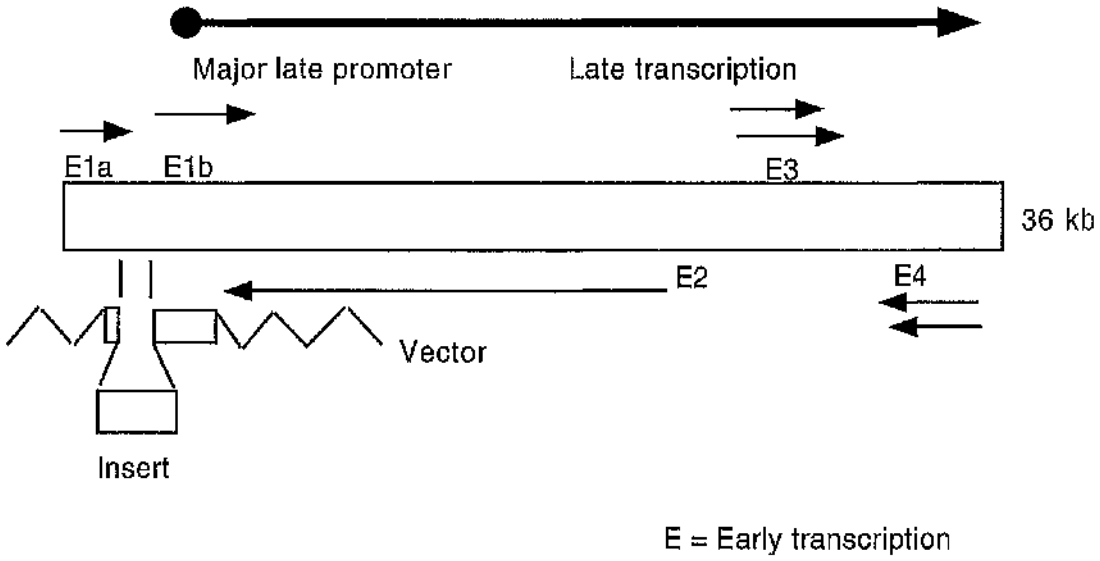

Figure 1.2: Adenovirus genome

Adenovirus genome showing the early and late transcription regions. First generation adenoviral vectors had the E1 region deleted making them replication defective.

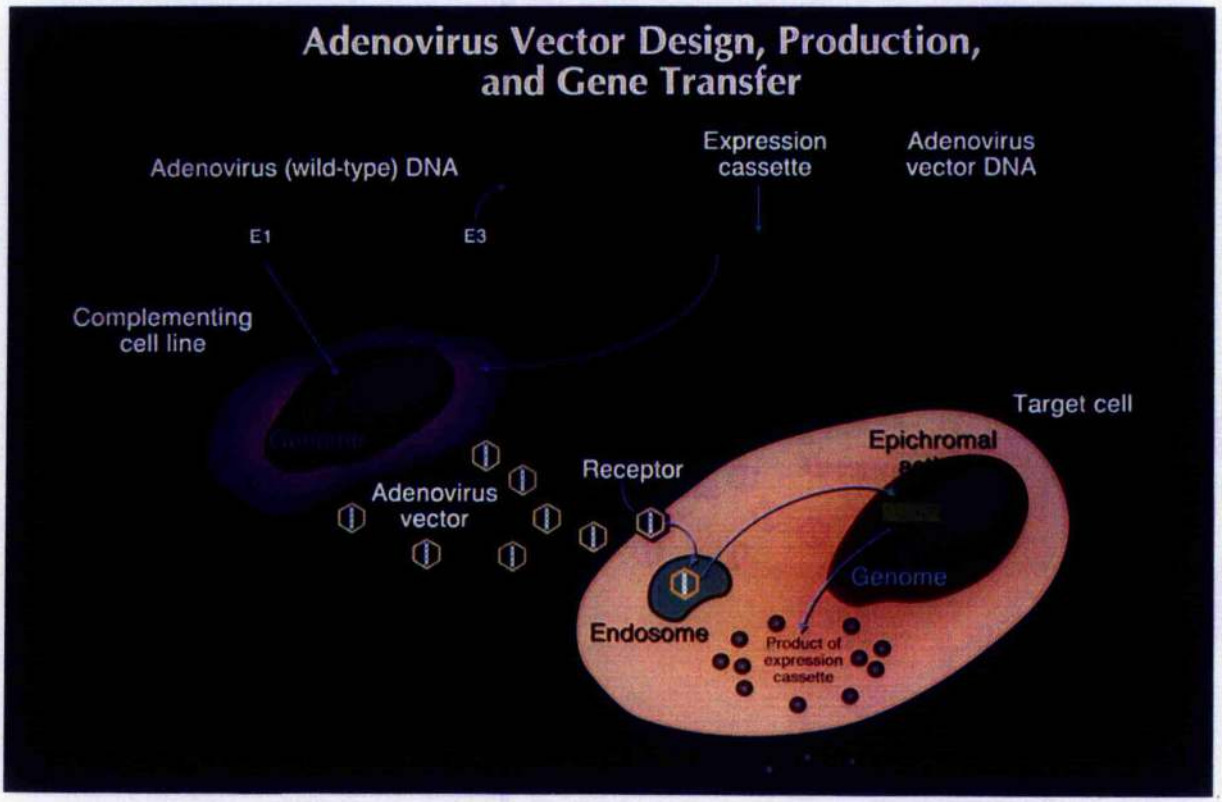

Figure 1.3: Adenoviral vector system

Adenovirus vector design, production and mechanism of gene transfer.

Chapter 2 : Design Of Experiments And Methods

Specific Aims of Study

The aim of this research was to examine the use of adenoviral vectors for gene transfer in the setting of heart transplantation. Access to the donor heart at the time of harvest provides a unique opportunity for genetic manipulation of this organ. Specifically, this study addressed the feasibility of gene transfer to the donor heart, the duration of transgene expression after transplantation and the feasibility of overexpressing a biologically relevant gene product, endothelial nitric oxide synthase (eNOS) in the transplanted heart.

Specific Aim 1: Hypothesis: Adenoviral-mediated gene transfer can be used to genetically modify the donor heart.

The aim of this section was to determine the optimal conditions for gene transfer to the donor heart. We first examined a dose response curve for adenoviral-mediated gene transfer at 4 ^{0} C (A below) and then studied the effect of varying the conditions of exposure to the adenoviral vector (B below). Heterotopic syngeneic transplants between Lewis rats were used in these experiments.

A) In this section, the optimal dose of virus for gene transfer at 4°C were studied. The following concentrations of virus were used: 10^{7}, 10^{8}, and

10^9 plaque forming unit per ml (pfu/ml). Donor hearts were transduced with each of these concentrations of virus for 1 hour at 4°C. These donor hearts were then transplanted into recipient animals. Four days after transplantation, the animals were sacrificed, the transplanted and recipient native hearts were harvested and analysed for transgene expression. Sham transduced syngeneic transplants were used as controls.

B) In this section the effect of varying conditions of adenovirus delivery on transgene expression were assessed. At the time of donor heart harvest, gene transfer, using the optimal concentration as determined by the experiments in section (A), in varying conditions were examined. Four days after transplantation, the animals were sacrificed, the transplanted and recipient native hearts assessed for transgene expression.

Specific Aim 2: Hypothesis: Temperature affects efficiency of gene transfer.

The aim of this section was to determine the effect of temperature on the efficiency of gene transfer in endothelial cells. *In vitro* experiments were devised in which human umbilical vein endothelial (HUVEC) cells were transduced with an adenoviral vector encoding for the β-galactosidase gene (AdLacZ) at 4^0C, 10^0C and 37^0C.

Specific Aim 3: Hypothesis: Prolonged transgene expression in the transplanted heart may be possible after *ex vivo* adenoviral-mediated gene transfer.

The aim of this section was to examine the duration of transgene expression in the transplanted heart having determined the optimal dose and conditions in Specific Aim 1. We examined the duration of transgene expression following a) syngeneic transplantation in non-immunosuppressed animals (A below), b) syngeneic transplantation in immunosuppressed animals (B below), and then c) allogeneic transplantation in immunosuppressed animals (C below) (Donors - Brown Norway; recipients - Lewis rats).

A) To determine the duration of transgene expression in non-immunosuppressed syngeneic animals, donor hearts were transduced at the time of harvest by a dose of AdLacZ (in conditions determined by Specific Aim 1) and then transplanted into syngeneic recipients. After transplantation, animals were harvested at the following time points: 1, 4, 8, and 12 weeks. Six animals were used for each time point. Transplanted hearts were harvested at these timepoints and analysed for transgene expression.

B) To determine the duration of transgene expression in immunosuppressed syngeneic animals, donor hearts were transduced at the time of harvest by a dose of AdLacZ (in conditions determined by Specific Aim 1) and then transplanted into syngeneic recipients. After transplantation, animals were treated with cyclosporine by orogastric injection, 10 mg per kg each day for 14 days followed by 5 mg per kg per day thereafter[75,76]. Animals

were sacrificed at the same time points as described in part A above. Transplanted hearts were analysed for transgene expression.

C) To determine the duration of transgene expression in immunosuppressed allogeneic animals, donor hearts (from Brown Norway rats) were transduced at the time of harvest by a dose of AdLacZ (in conditions determined by Specific Aim 1) and then transplanted into allogeneic recipients (Lewis rats). After transplantation, animals were treated with cyclosporine by orogastric injection, 10 mg per kg each day for 14 days followed by 5 mg per kg per day thereafter by orogastric injection. Animals were sacrificed at the same time points as described in part A above. Transplanted hearts were analysed for transgene expression.

Specific Aim 4: Hypothesis: Nitric oxide synthase (NOS) can be overexpressed in the transplanted heart using adenoviral-mediated gene transfer.

The aim of this section was to study the feasibility of transferring biologically relevant genes to the donor heart. Two groups of heterotopic syngeneic transplants between Lewis rats were used in the experiments.

Using the optimal dose and conditions as determined by Specific Aim 1, twelve rat heart transplantations were carried out in each group. Group 1 received an adenoviral vector encoding for the bovine endothelial nitric oxide synthase gene (AdeNOS) and Group 2 received AdLacZ as controls. The

transduced hearts were transplanted into recipient animals. These animals were sacrificed 4 days later. Successful gene transfer and transcription were assessed by the detection of DNA and mRNA of the transgene by polymerase chain reaction (PCR) and reverse transcriptase-polymerase chain reaction (RT-PCR) respectively. The 5' probe used in these analysis included viral sequences that would allow differentiation between endogenous eNOS and transgene expression of eNOS. Transgene expression at protein level was detected by immunohistochemical staining. Antibodies to endothelial bovine NOS were used which did not cross react with the inducible isoform of NOS. Activity and functional assessment of recombinant eNOS were made using an activity assay and isolated heart preparation respectively.

Adenoviral Vectors

Generation, Propagation and Purification of Adenoviral Vectors:

An adenoviral vector encoding for the β-galactosidase gene (AdLacZ) under the control of the Cytomegalovirus (CMV) promoter was provided by Dr J M Wilson from the Institute of Gene Therapy, University of Pennsylvania. This vector had been used by the present laboratory to transduce a number of cell lines *in vitro* and the rabbit carotid artery *in vivo* with high efficiency. Cells transduced with this vector stained blue when

exposed to X-Gal chromogen. This allowed the efficiency of gene transfer to be assessed. This vector was used in all the studies described in Specific Aims 1, 2, 3 and 4 of this thesis.

An adenoviral vector expressing the bovine endothelial nitric oxide synthase (AdeNOS) gene was generated by Dr O'Brien (Mayo Clinic). The cDNA for eNOS was cloned into a subsegment of the adenoviral genome which was itself cloned into a plasmid. The eNOS gene in this case was under the control of the CMV promoter. The resulting construct was cotransfected with dl309 (adenoviral DNA) into 293 cells. Recombinant adenovirus was generated as a result of homologous recombination. Viral plaques were selected and propagated in 293 cells. Viral DNA was enriched by Hirt extraction and screened by restriction mapping and polymerase chain reaction for the presence of eNOS DNA. Viral preparations were generated by infecting confluent monolayers of 293 cells with viral stock at an MOI (multiplicity of infection) of 10 pfu/cell. Virus was purified by double cesium chloride gradient ultracentrifugation and dialyzed against 10 mmol/L Tris, 1.0 mmol/L $MgCl_2$, 1.0 mmol/L HEPES, and 10% glycerol for 4 hours at 4^0C. Viral titre was determined by plaque assay. eNOS activity was confirmed by positive NADPH diaphorase staining in confluent 293 cells transduced with AdeNOS. This vector has been used successfully to transduce porcine coronary smooth muscle cells *in vitro*. The functional nature of the transgene was demonstrated by increased production of nitrite and cyclic GMP levels by

cells transduced with AdeNOS in comparison to cells transduced with AdLacZ.

Adenoviral vectors used were replication incompetent due to E1a deletion. However, cell lines containing this region of the adenovirus genome could complement E1a-deleted virus and support productive infection. The human 293 cell line was derived for this purpose by the introduction of sheared fragments of Adenovirus 5 (Ad5) DNA into an embryonic kidney carcinoma cell line [40]. This cell line contains the E1 region of the adenoviral genome and can therefore provide protein products in trans, thus allowing for the propagation of replication defective adenoviral vectors.

During the propagation of the adenoviral vector, replication competent virus may be generated. Therefore in order to exclude wild-type contamination, the defectiveness of the AdeNOS for replication was tested by adding the virus (10^8 pfu/ml) to a monolayer of diploid human embryonic lung fibroblasts (60 mm dish). Replication competent viruses at an MOI of ≥ 10 produce a cytopathic effect and destroy the monolayer in < 3 days. Infection with the AdeNOS at a comparable MOI produced no observable cytopathic effect after 5 days. This showed the replication defectiveness of the vector. Furthermore, polymerase chain reaction for detecting E1A was performed on all the adenoviral vector preparations. This was negative in all the adenoviral vector preparations tested but was positive in dl309 which was used as a positive control. Both methods described excluded wild-type contamination of the adenoviral vectors. Wild-type contamination of recombinant adenoviral

vectors may lead to confounding factors which may affect laboratory experiments. In addition, it may have biosafety implications in both laboratory and clinical settings.

The adenoviral vectors were stored at -70^{0}C in 0.01M Tris, 0.01M MgCl, and 10% glycerol. For use in experiments, the viral solution was thawed and diluted in 2% fetal calf serum in 199 medium to the required concentrations. It was noted that repeated thawing and freezing of the viral solution led to reduced infectivity of the vector.

Heterotopic Abdominal Heart Transplantation in a Rat Model

Animals

Lewis rats (Rtl^l) were used as donors and recipients for syngeneic transplants. This combination did not result in rejection of the transplanted organs. For allogeneic transplants, Brown Norway (Rtl^n) rats were used as donors and Lewis rats as recipients. The Brown-Norway to Lewis strains combination represented a severe immunological mismatch and donor hearts were normally rejected in approximately seven days[77].

All animals received humane care in compliance with the "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 (NIH Publication No. 86-23, revised 1985).

Heterotopic Heart Transplantation

Rat heterotopic abdominal heart transplantation is a well established animal model in transplantation research. Heterotopic abdominal heart transplantation has been used extensively as a convenient and reproducible technique to study many aspects of the host immunological responses to allograft tissues. It is technically uncomplicated and has the advantage of easily determining graft function by palpation of the beating heart. However, as the left ventricle is not loaded in this model, functional studies and results should be interpreted with caution. Although functional impairment of the left ventricle has been shown at 1 week after transplantation, for immunological and gene transfer research, heterotopic abdominal heart transplantation remains a valuable model that is widely used. This model proves to be effective, cost and time efficient because the surgical procedure is simple, relatively fast and technically successful. With practice, appropriate microsurgical skills and equipment, success approaches 95%. It was for these reasons that this research protocol adopted this animal transplantation model.

Preliminary Training in Microsurgery

The applicant's initial exposure to microsurgery was in the Mayo Microvascular Training course. Instructions were given concerning the care and use of the operating microscope and standard microsurgical instrumentation. Specifically, the training was in the techniques of arterial to arterial, venous to venous and arterial to venous anastomosis of femoral vessels in rats.

Following this, the training protocol for heterotopic abdominal heart transplantation in the rat model was approved, where the necessary skills for performing the operation with low mortality were acquired.

Techniques of Heart Transplantation and Gene Transfer:

Donor:* *Anaesthesia and operation

The donor animal was anaesthetised with an intraperitoneal injection of 5 mg sodium per 100 gm body weight of pentobarbital (Pentobarb, Abbott Laboratories, N Chicago, Il). The intraperitoneal route was chosen to achieve a swift and effective anaesthesia of the donor animal which was euthanised on harvesting the heart. The chest was shaved and the animal intubated by direct vision with a 14G plastic cannula (Figure 2.1) and ventilated with 28% oxygen using a Harvard Rodent Ventilator (model 683, Harvard Apparatus, S Natick, Mass). The animal was placed supine and all four limbs were spread out and secured onto the operating platform with adhesive tapes. The chest and upper

abdomen were cleansed with betadine solution and the area prepped with disposable drapes.

A median sternotomy was made and a retractor was used to spread the chest, exposing the pericardium. The pericardium was then incised and the heart exposed. The rat was then heparinised with 300 units of aqueous heparin injected into the inferior vena cava. Silk slings were placed around the innominate artery and the distal arch of the aorta (Figure 2.2). A 21 gauge IV cannula was inserted into the innominate artery and directed into the aortic root. The cannula was secured with the silk tie around the innominate artery. The cannula was then connected to the cardioplegic infusion line. Next, the venae cavae and pulmonary veins were ligated en block with a single 4-0 silk. The pulmonary artery was divided and the distal ascending aorta occluded using the silk sling placed earlier. The donor heart was arrested with an infusion of cold, dilute St Thomas' cardioplegic solution delivered using a syringe pump at a rate of 0.44 ml/min. Following satisfactory cardioplegic arrest, the donor heart was excised and placed in a cold cardioplegic solution at 4^0C. A viral solution was injected into the aortic root using the indwelling cannula with the distal aorta tied and the heart was then stored in cold cardioplegic solution until transplantation (Figure 2.3). During this time, the viral solution was in contact with the donor heart coronary system.

Recipient: *Anaesthesia and operation*

Recipients, weighing 250-375 grams, were anaesthetised with halothane and O_2. The recipient animal was intubated using a 14 gauge IV cannula and ventilated with halothane and oxygen.. This method of anaesthesia allowed for a more predictable time course in recovery from anaesthesia in the post-operative period.

After prepping and draping, a vertical midline abdominal incision was made to expose the abdominal aorta and inferior vena cava. The two vessels were separated over a segment below the renal vessels. The aorta was clamped proximal and distal to the site of anastomosis. The anterior wall of the aorta was lifted with a pair of forceps and a 2-3mm longitudinal incision was made. 10/0 ethilon sutures were placed as stay sutures at each end of the incision in preparation for the anastomosis of the donor aorta. Heterotopic heart transplantation was performed by anastomosing the donor aorta to the side of the recipient's infrarenal aorta and the donor pulmonary artery to the recipient's inferior vena cava using continuous 10/0 ethilon suture (Figure 2.4). The inferior vena cava tie was first released followed by reperfusion from the aorta by the release of the aortic clamp (Figure 2.5). In most instances, the transplanted heart started beating spontaneously within 30 seconds. Haemostasis was then secured and the abdominal wound closed with 3-0 vicryl suture. The halothane was switched off and the animal extubated when breathing spontaneously. The animal was then recovered in a cage with supplementary oxygen via a face mask in a warm room. Intramuscular

torbugesic was given as analgesia. In compliance with the biosafety guideline, the transplanted animals were housed in a dedicated room with biosafety level 2 approval. Viability of the transplanted heart was checked by daily palpation of the beating heart.

Adenoviral vector delivery

A volume of 350μl of viral solution (diluted in 2% fetal calf serum in 199 medium) was used to transduce each donor heart in all experiments. Sham animals injected with the same volume of medium alone were used as controls. The volume for coronary injection in the rat heart was determined using medium stained with Evans Blue. The blue-stained medium was infused into the aortic root via a cannula as described above This technique was used in all the experiments in this study. Only one investigator performed all the operations in order to maintain consistency and to avoid major differences in infusion pressure. The volume of medium infused resulting in the effluent from the pulmonary artery becoming fully stained was determined in 5 animals and the mean volume (350 μl) used in all further experiments. This was done to ensure that the whole coronary system was exposed to the studied viral solution.

Specimen Preparation

The transplanted hearts were harvested at specified time-points in the protocol for histochemical analysis. The transplanted heart was removed and

the coronary system flushed with PBS (Phosphate buffered saline). The midventricle was located by placing the explanted heart next to a ruler. The heart was then cut with a razor blade to give a midventricular cross section of approximately 5 mm in thickness. This cross section was embedded in OCT compound (Elkhart, Ind) and snap frozen in an isopentane bath cooled in liquid nitrogen. Initially, the ventricular segments in OCT compound were snap frozen in liquid nitrogen directly. This resulted in an unacceptable degree of cytoarchitectural disruption of the histological sections. This was most likely due to the extreme temperature. However, the use of an isopentane bath in liquid nitrogen appeared to allow for a more controlled freezing of the specimens which resulted in better preservation of the cytoarchitecture.

Five 5μm thick cryostat sections were then cut at 25μm intervals. The 25μm intervals was chosen to provide a reasonable degree of sampling of the mid-ventricular segment for transgene expression (Figure 2.6).

Histological Analysis

Staining for β-galactosidase Expression

In **Specific Aims 1 and 2**, the adenoviral vector coding for the reporter gene β-galactosidase, an enzyme present in Escherichia Coli, was used. The ease and rapid in situ detection of β-galactosidase by 5-bromo-4-chloro-3-

indoyl-β-D-galactopyranoside (X-Gal) staining allowed for an unambiguous and precise appreciation of adenoviral-mediated gene transfer. X-Gal is a substrate for β-galactosidase and the reaction releases the indoyl component giving the unmistakable blue staining of the transduced cells. Cells which had been transduced with a vector encoding the gene for β-galactosidase could be identified *in vitro* or *in vivo* by staining with X Gal. However, one caveat which should be borne in mind is the fact that normal tissue may stain blue if left exposed to the chromogenic substrate for a prolonged period of time. Thus, it is essential to limit the time of exposure to the chromogenic substrate in order to avoid spurious results. In all experiments described in this thesis staining for β-galactosidase was performed for four and a half hours.

Preparation of X Gal solution:

X Gal solution is prepared as follows:

To make 10 mls of X-Gal solution mls,

add 9 mls of distilled water

250µl of 2M Tris-HCl solution.

500µl of Ferriferrocyanide (2.5mM) solution (freshly made)

30µl of 5M NaCl solution

10µl of 1M MgCl2 solution

125µl of X-gal (40 mg/ml) (Added last).

Staining with X Gal

5μm sections of hearts were fixed in 1.25% PBS/glutaraldehyde for 10 minutes at 4°C and rinsed three times with Phosphate buffered saline (PBS). The sections were then immersed in a solution of X-Gal for 4.5 hours at 37°C. The specimens were then rinsed in PBS and counterstained with eosin which gave a contrasting red stain. Blue stained cells indicated the presence of β-galactosidase expression.

H & E staining

For assessment of rejection, Haemotoxylin and Eosin (H & E) staining was done for all transplanted hearts in the Specific Aim 3 study. The rejection scoring was done by an experienced cardiac transplantation pathologist who was blinded to the identification of the specimens. Rejection score was in accordance with the Heart Rejection Working Group [78].

Molecular and immunohistochemical staining

In Specific Aim 4, the detection of successful transduction of the transplanted heart with eNOS gene was done using molecular and immunohistochemical techniques. As these techniques were used only in the Specific Aim 4 study, full details will be given in the relevant Chapter in the thesis.

Isolated Heart Perfusion: Langendoff Preparation

For functional studies in Specific Aim 4, the isolated heart perfusion model was used. The applicant was solely responsible for setting up this model and validating it (Figure 2.7). The guidelines were from the book "The Isolated Perfused Heart: According to Langendorff" by H. J. Doring and H. Dehnert.

Studied animals were anaesthetised by intraperitoneal injection of 5 mg sodium per 100 gm body weight of pentobarbital (Pentobarb, Abbott Laboratories, N Chicago, Il). The transplanted hearts were arrested with cold saline at 4 ^{0} C, quickly excised and then mounted onto the aortic cannula of the Langendorff apparatus and perfused immediately with Krebs buffer (containing, in mmol/l: glucose 11.1, NaCl 118.3, KC1 4.7, $MgSO_4$ 1.2, KH_2P0_4 1.2, $NaHC0_3$ 25.0, $CaC1_2$ 2.5, Edetate Calcium Disodium 0.026, at pH 7.4 when gassed with 95% 0_2 plus 5% CO_2). This expeditious manoeuvre was achieved by using a small vascular clip to secure the aortic root to the perfusing cannula. Once the heart was perfused and beating vigorously, the aorta was then secured with a silk tie and unwanted tissues were trimmed. Perfusion pressure was then set at 60mmHg. All hearts were paced using a Grass SD (Pacing Grass SD9 Stimulator, Quincy, MA) via the right atrium at 320 beats/min. The environment around the heart was maintained with the use

of a water-warmed jacket. An in-line flow probe (Transonic System Inc, Ithaca, NY) was position above the aortic root to measure coronary flow. This equipment has been calibrated by the manufacturer and gave an accuracy to ± 0.5 ml/minute. The left atrium was then opened widely to facilitate the introduction of an intraventricular balloon. The size of the intraventricular balloon (size 4, Radnotti, CA) was chosen to fit into the left ventricular chamber. This was connected via a plastic cannula to a three way tap. One limb of the tap was connected to a Millar catheter (Millar Instrument Inc. Houston, Texas) for left ventricular measurement while the third limb was connected to a calibrated syringe filled with saline. This allowed the intraventricular balloon pressure to be varied. Following 15 minutes of stabilisation, left ventricular function curves were constructed by progressively increasing the volume of the balloon to achieve end-diastolic pressures. Following the assessment of cardiac function, the LVEDP was maintained at the optimal level and was heart allowed to stabilise again for 5 minutes. Then coronary flow was measured at increasing time intervals (1, 2, 3, 4, 5, 10, 15, 20, 25 minutes)

Data Collection and Analysis

For quantitative analysis of gene transfer, the total number of positive staining cells per section were counted under magnification (x 100) and the mean value was calculated from five sections in each heart. All sections were reviewed by one investigator blinded to the identification of the sections. Furthermore, random slides were examined and counted by an independent person and results compared.

Results were expressed as median (and range) or mean (and standard deviation) of the number of positive staining cells per section in each group. The small sample size used in these experiments precluded the formal testing of the data for Gaussian distribution. Therefore, the characteristics of the data determined the statistical tests used. If the data suggested a non-Gaussian distribution and variances were unequal, despite log transformation, non-parametric tests were used. If the data suggested a Gaussian distribution and variances were equal, parametric tests were used. In all instances, a P value of < 0.05 was considered significant.

Biosafety Issues: Precautions for working with adenoviral vectors.

The use of biological vectors in gene transfer studies raises important biosafety issues. Adenovirus serotype 5 were used in all the experiments in this thesis.

There are more than 40 different human serotypes of adenovirus identified, of which types 2 and 5 have been extensively studied and used in gene transfer studies. The wild type adenovirus is known to cause mild respiratory infections which follows a self-limiting course in humans. None of the serotypes have been shown to have oncogenic potential. Although these vectors had been tested and shown to be replication defective, caution in handling these agents is necessary, in view of the complementary DNA (cDNA) inserts present in the recombinant adenovirus vectors. There is a theoretical risk of insertional mutagenesis in targeted organs. Also potential interaction with wild type adenovirus encountered in the environment may lead to a reversal of the replicative status of the vectors.

There are four different defined biosafety levels which consist of combinations of laboratory practices and techniques, safety equipment and laboratory facilities. Each combination is specifically appropriate for the operations performed, the route of transmission of the infectious agents, and for the laboratory function or activity. The recommended biosafety level(s)

for organisms represent those conditions under which the agents can ordinarily be safely handled.

Working with adenoviral vectors represented a Biosafety level 2 risk (BSL-2). In brief, Biosafety level 2 practices, equipment and facilities applicable to clinical, diagnostic, teaching and other facilities in which work is done with the broad spectrum of indigenous moderate-risk agents present in the community and associated with human disease of varying severity. With good microbiological techniques, these agents can be used safely in activities conducted for the open bench, provided the potential for producing splashes or aerosols is low.

Therefore, in accordance with NIH (National Institute of Health) guidelines, all work with adenoviral vectors were performed in a BSL-2 (Biosafety level 2) facility. However, due to the recombinant nature of these vectors, additional precautions as detailed below were adopted during these experiments.

Laboratory:

All adenovirus vector generation, propagation and purification were performed in a specified laboratory. This facility has a tissue culture area and has been certified as a BSL 2 facility by the Mayo Clinic Institutional Biosafety Committee. All tissue analysis was performed in laboratories that had limited access while experiments were in progress. Biohazard warning signs were posted and precautions for the disposal of sharp objects exercised.

A biosafety cabinet was used for all manipulations involving recombinant adenoviral agents. Laboratory coats and gloves were worn at all times in the work area and removed prior to leaving. All laboratory waste were autoclaved prior to disposal.

Animal Facilities:

All animal procedures were performed in BSL 2 approved rooms. Heart transplantations were performed in the designated microsurgery room. All surgical drapes were disposable. After surgery, rats were housed in a designated room and access to the area was restricted when animals transduced with adenoviral vectors were present. Doors to the animal room opened inward, self-closing, and were kept closed when experimental animals were present. A hazard warning sign was posted, incorporating the universal biohazard symbol, in which the presence of animals with adenoviral vectors were documented. All wastes from the animal room were autoclaved prior to disposal. Infected animal carcasses were incinerated after being transported from this room in leakproof, covered containers.

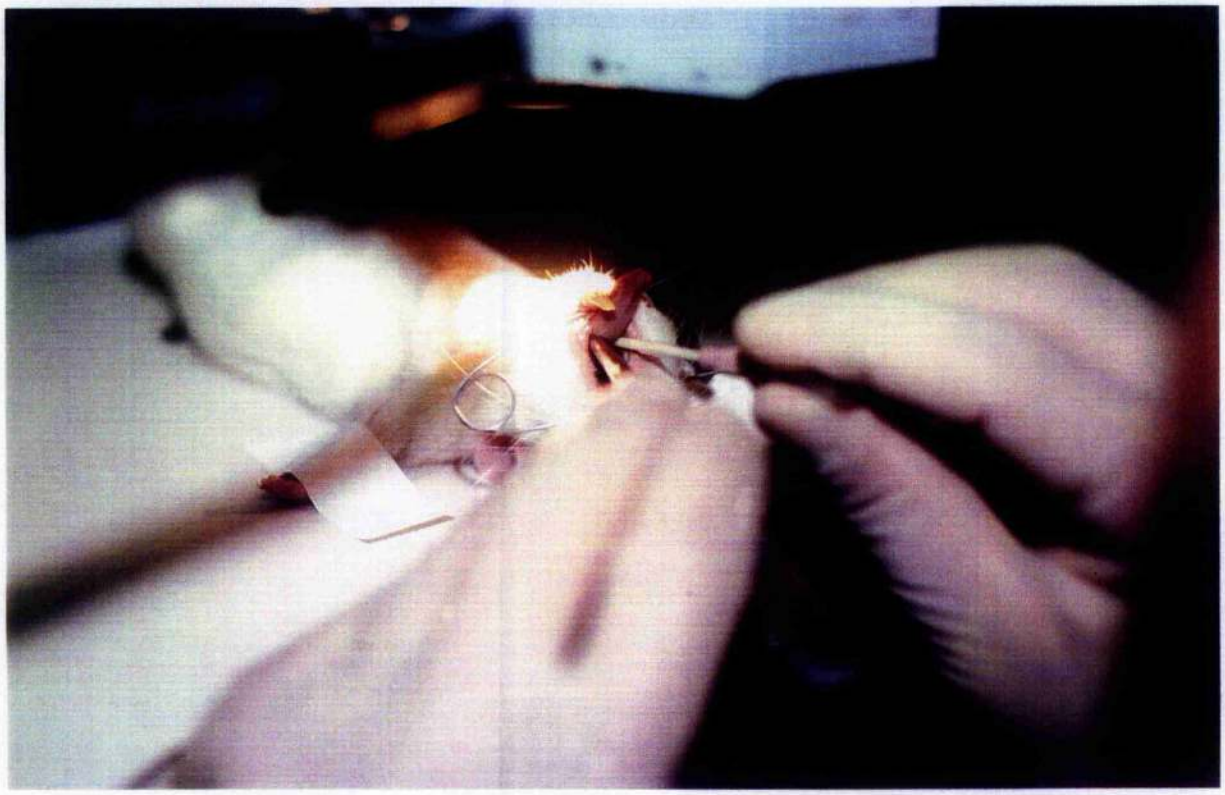

Figure 2.1: Intubation for ventilation

A 14G plastic cannula was used to intubate the animal in preparation for ventilation.

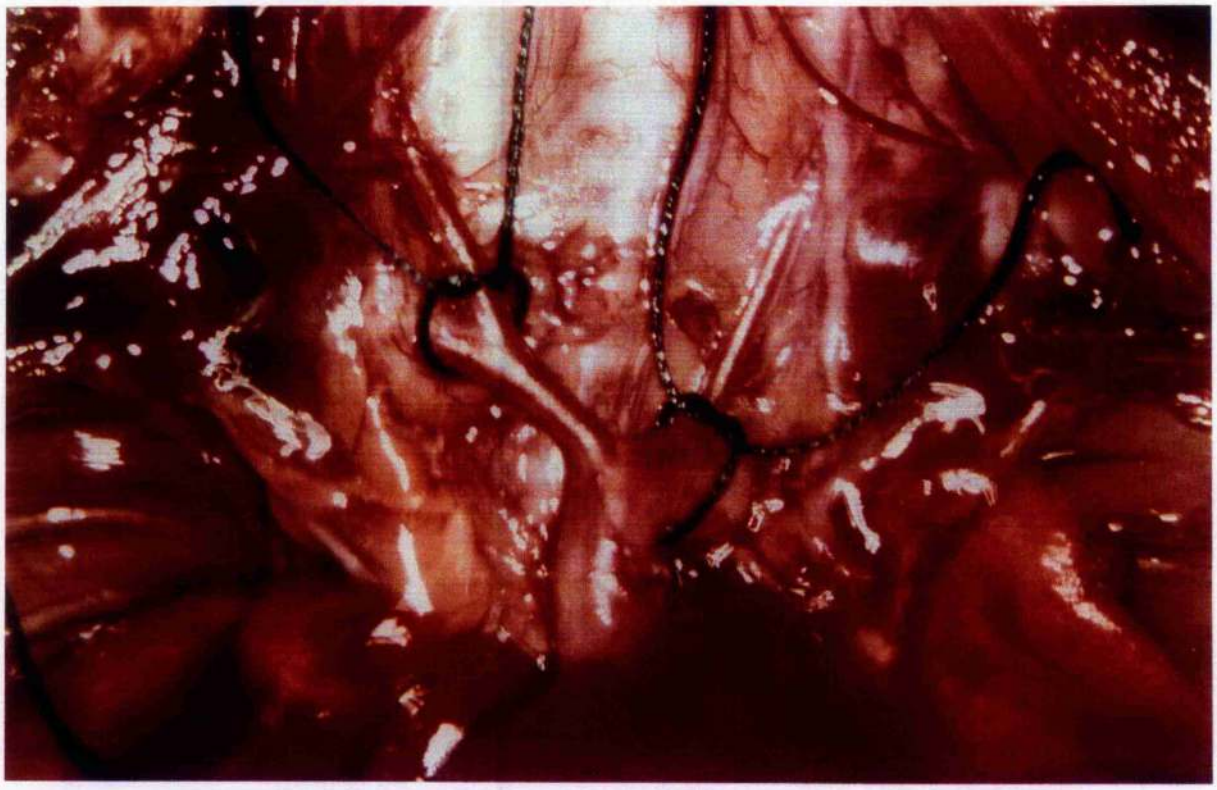

Figure 2.2: Aorta in preparation for cannulation

Silk slings were placed around the innominate artery and the aorta just distal to it. The innominate sling was used to secure the cardioplegia cannula and the aortic sling used as a cross clamp.

Figure 2.3: Intracoronary viral infusion

Viral infusion into aortic root via indwelling cannula with the heart in cold cardioplegic solution.

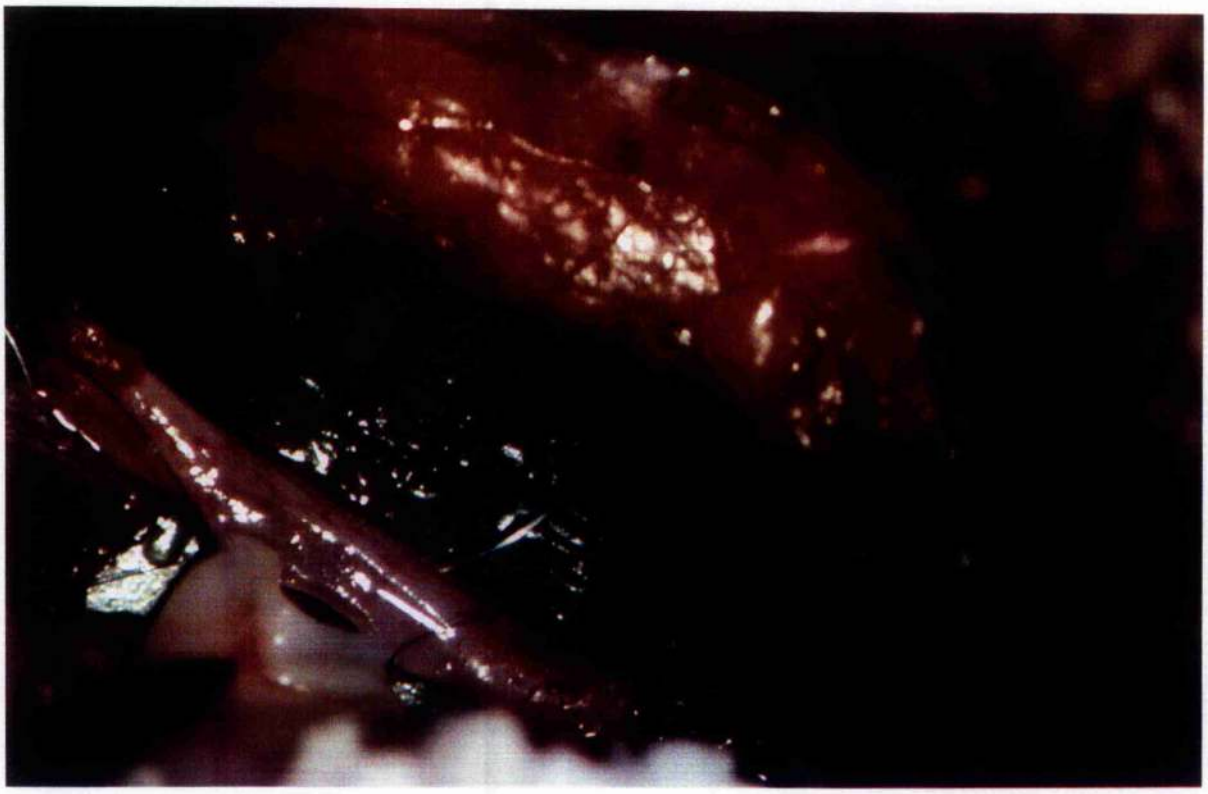

Figure 2.4: Heterotopic heart transplantation.

The ascending aorta of the donor heart is anastomosed to the abdominal aorta of the recipient using microsurgical techniques and instruments.

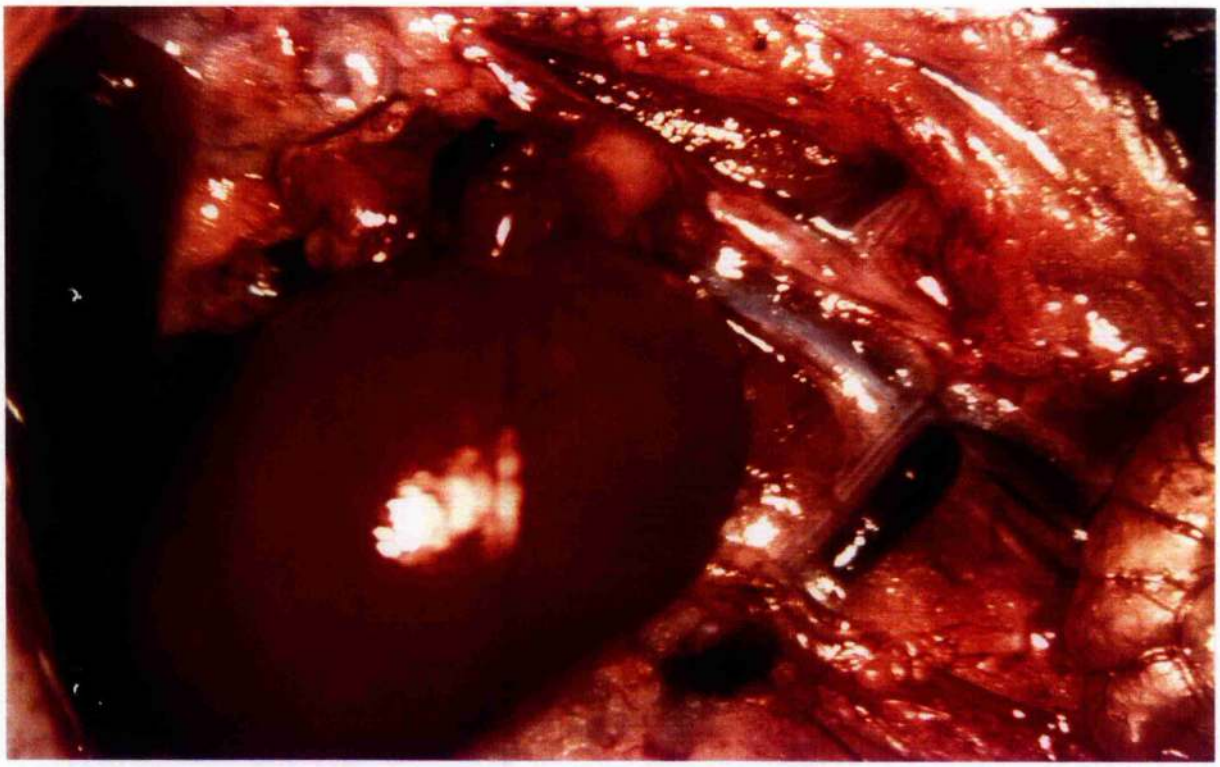

Figure 2.5: Reperfusion of transplanted heart

On completion of the vascular anastomoses, the transplanted heart was reperfused by releasing the aortic tie.

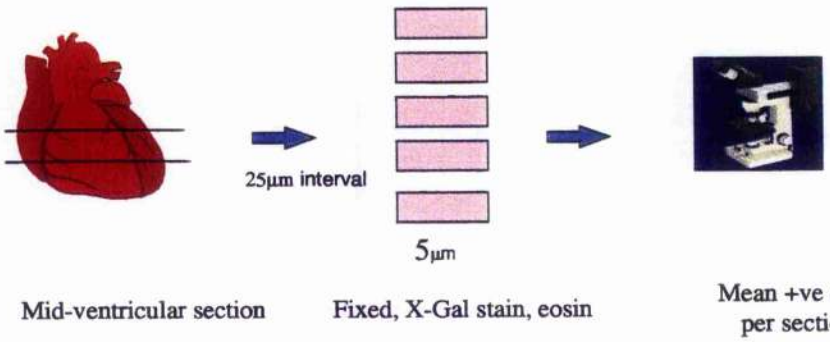

Figure 2.6: Assessment of gene transfer

Assessment of heart following gene transfer by light microscopy. Five slides of the mid-ventricular section were examined and the mean total of positive staining cells were counted and expressed as mean number of cells per section.

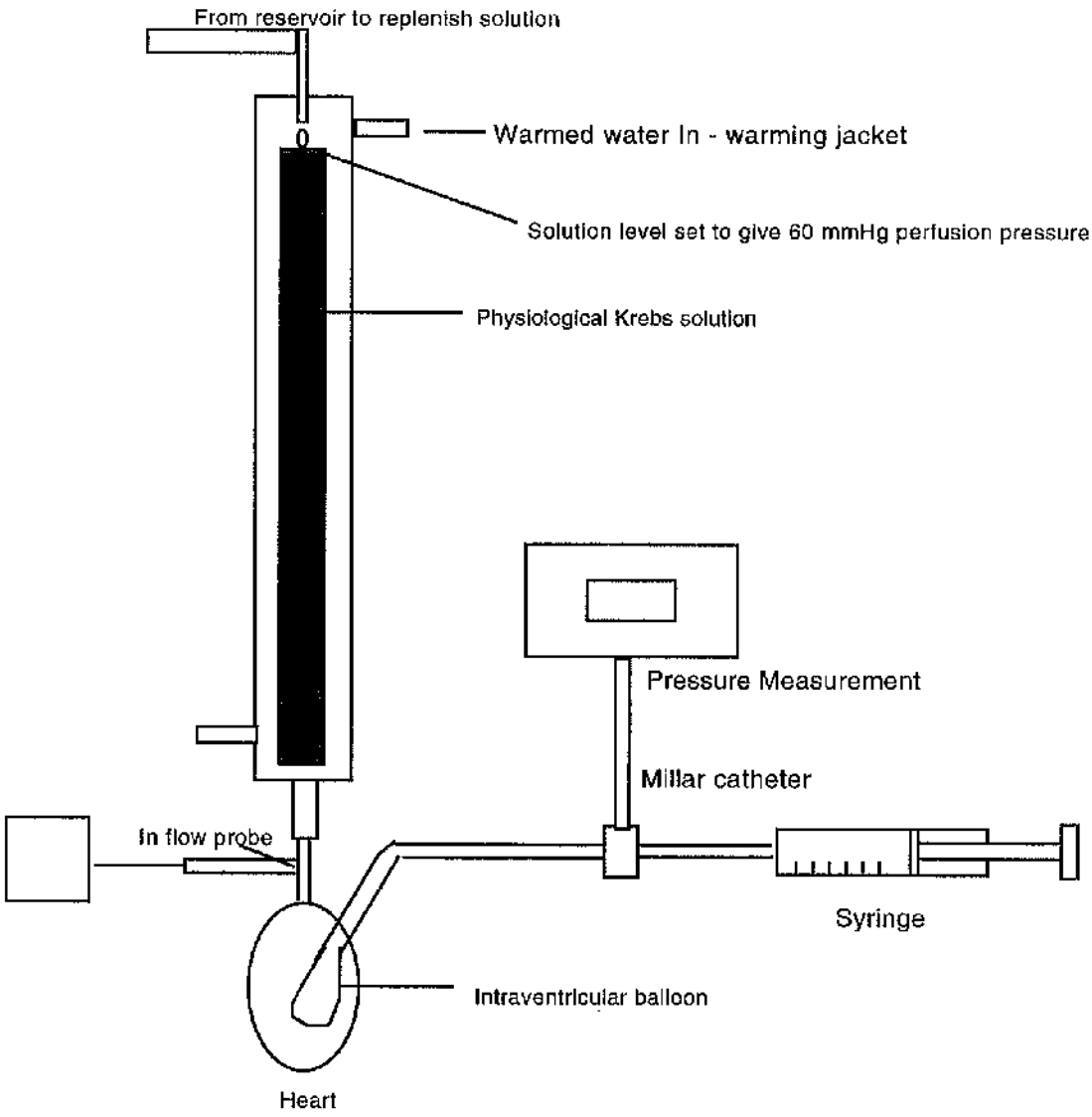

Figure 2.7: Schematic representation of the isolated heart perfusion model

This model of isolated heart perfusion was used to assess the functional effects of overexpression of eNOS in transplanted hearts.

Chapter 3 : Adenoviral-Mediated *Ex Vivo* Gene Transfer To The Transplanted Heart: Effects Of Varying Vector Delivery Conditions.

Introduction

Heart transplantation is an accepted treatment for end stage cardiac disease in selected patients [79,80]. However, limitations to clinical heart transplantation include acute and chronic rejection, infection, and the side effects of immunosuppressive therapy. Gene therapy offers the potential for modifying these processes at the molecular level. In previous *in vivo* studies of vascular gene transfer, one challenge has been the need to interrupt blood flow to obtain luminal administration of recombinant DNA [31,69]. This limits the time in which the vector is in contact with target cells. Heart transplantation may be a more ideal setting for gene transfer as a more prolonged dwell time can be achieved during the necessary period of ischaemia.

Adenoviral vectors have been used to achieve efficient transfer and expression of recombinant genes in different vascular beds both *ex vivo* and *in vivo* [58,81]. One advantage of these vectors for gene transfer to the vasculature is the ability to transduce non-replicative cells. Adenoviral vectors can be grown to high titre and are not incorporated into the host cell genome [81] rendering these vectors safer to work with. Earlier studies have demonstrated the feasibility of gene transfer to the transplanted heart with liposomal [65] and adenoviral vectors [68]. However, the conditions for optimal gene transfer to the transplanted heart using adenoviral vectors have not been defined. This series of experiments was designed to investigate the varying conditions for gene

transfer in the heart transplant setting. Specifically, we sought to examine the effect of viral dose, the presence of virus in the donor heart during warm ischaemia and the effect of increasing coronary distension by clamping the pulmonary artery during viral infusion.

Methods

Animals

Inbred male Lewis rats weighing 250 to 320 grams were used as syngeneic donors and recipients. All post-operative rats received analgesia and were allowed to recover with supplementary oxygen in a warm environment.

Adenovirus Vector

A first generation E1a-deleted (replication-defective) and non-nuclear targeted adenovirus vector encoding for β-galactosidase (ΛdLacZ) under the control of the CMV promoter was used in these experiments. The recombinant virus was propagated, isolated and purified as described in Chapter 2. Viral titres were determined by plaque assay and expressed as plaque forming unit per ml (pfu/ml).

Operation and Gene Transfer

Abdominal heterotopic heart transplantation using standard microsurgical techniques was performed [82]. The technical details have been described in Chapter 2. A volume of 350μl of viral solution (diluted in 2% fetal calf serum in 199 medium) was infused over 5 seconds into the coronary arteries via the aortic root. After 60 minutes of cold storage at 4°C, all donor hearts were heterotopically transplanted into the recipients. Function of the grafts was assessed daily by palpation of the beating transplanted heart.

Experimental Groups

In **experiment A,** the pulmonary artery was left open during viral infusion and at the end of 60 minutes the virus was flushed out with repeat infusion of cold cardioplegic solution prior to transplantation. A dose response experiment was carried out to determine the optimal viral concentration for gene transfer under these conditions. Viral concentrations of 1×10^{7} pfu/ml (n=5), 1×10^{8} pfu/ ml (n=5), 1×10^{9} pfu/ ml (n=6), and medium only as control (n=5) were used. The most effective viral concentration was then used in subsequent experiments (below) to compare the efficiency of gene transfer under different conditions.

In **experiment B,** using the viral concentration as determined in the initial experiment (1×10^{9} pfu/ml), the pulmonary artery was clamped during

viral infusion and the virus was not flushed out with cold cardioplegic solution at the end of 60 minutes prior to performing heart transplantation (n = 6).

Having determined the above improved conditions for gene transfer, a further dose response study (**experiment C**) was carried out with two additional viral concentrations (1×10^{8} pfu/ml and 1×10^{10} pfu/ml, n = 4 for each group) to determine the optimal concentration for gene transfer under the conditions of **experiment B.**

To further investigate the conditions that resulted in efficient gene transfer in **experiment B**, further experiments were performed. In **experiment D**, the role of increased coronary distension during viral infusion alone on the efficiency of gene transfer was studied. A viral solution of 1×10^{9} pfu/ml (as determined in previous experiments) was infused into the aortic root with the pulmonary artery clamped, resulting in the coronary system visibly distended, and the virus was then flushed out with cold cardioplegic solution at the end of 60 minutes prior to performing heart transplantation (n = 6). This was compared with **experiment E**, in which the role of virus warm dwell during transplantation on gene transfer efficiency was evaluated. A viral solution of 1×10^{9} pfu/ml (as determined in previous experiments) was infused into the aortic root with the pulmonary artery open, and the virus was not flushed out with cold cardioplegic solution at the end of 60 minutes prior to performing heart transplantation (n = 5).

In addition, the aortic root pressure was measured, using a pressure transducer, during viral infusion with and without the pulmonary artery clamped.

Heart Retrieval and Histochemical Analysis

Four days after surgery, transplanted hearts were harvested and processed as previously described. Briefly, the explanted heart was cut into three sections, apical, midventricular and basal, with a razor blade. The location of the midventricular section was estimated using a ruler. The section was then snap frozen. Five 5μm thick cryostat sections were cut at 25 μm intervals in the mid-ventricular section. The specimens were fixed and stained in a solution of X-Gal, then counterstained with eosin. Blue stained cells indicated the presence of β-galactosidase expression. For quantitative analysis, the total number of positive staining cells per section were counted under magnification (x 100) and the mean value was calculated from five sections in each animal.

Statistical Analysis

Results were expressed as median (range and standard error) of the number of positive staining cells per section in each group. As the data did not follow a Gaussian distribution and variances were unequal, a non-parametric test (Kruskal-Wallis) of analysis of variance was performed to evaluate overall

group differences for more than two groups. If overall significance was present, Dunn's post hoc test was used for pairwise comparisons. For comparison between two groups, the Mann-Whitney test was used (Prism, GraphPad, San Diego, CA). A P value of < 0.05 was considered significant.

Results

Operative mortality was 6%. All transplanted hearts were beating at the time of harvest. The mean transplant time (during implantation of the donor heart) was 29.7 minutes (range from 25 minutes to 35 minutes). There were no differences in ischaemic times between the groups.

In the initial dose response study (experiment A), the median (range and standard error of mean, SEM) of the total number of positive staining cells for β-galactosidase per section was 0.2 (0 - 1.8, SEM 0.34) in the 1×10^{7} pfu/ml group, 1.8 (0 - 12.6, SEM 2.44) in the 1×10^{8} pfu/ml group and 17.8 (0.6 - 37.6, SEM 5.03) in the 1×10^{9} pfu/ml group ($p < 0.05$, 1×10^{7} pfu/ml vs 1×10^{9} pfu/ml) (Figure 3.1). All sham transplanted heart stained negative for β-galactosidase.

In experiment B, a combination of clamping the pulmonary artery during viral infusion with a period of viral warm dwell, by not flushing the coronary system, was evaluated. Coronary distension was visibly greater than

when the pulmonary artery was clamped during the viral infusion. The median (range, SEM) of the total number of positive staining cells for β-galactosidase was 238.1 (32 - 608.2, SEM 91.48) in the 1×10^{9} pfu/ml group. This resulted in a significant increase in gene transfer ($p < 0.005$) compared with the 1×10^{9} pfu/ml group in experiment A (Figure 3.2).

Having determined that experiment B represented dramatically improved conditions for gene transfer, a further dose response evaluation (experiment C) was carried out with two additional viral concentrations (1×10^{8} pfu/ml and 1×10^{10} pfu/ml) under the same conditions as experiment B. The median (range, SEM) of the total number of positive staining cells for β-galactosidase was 0.4 (0 - 0.8, SEM 0.18) in the 1×10^{8} pfu/ml group and 276 (109 - 564.2, SEM 108.7) in the 1×10^{10} pfu/ml group. There was no difference in efficiency of gene transfer between 1×10^{9} pfu/ml and 1×10^{10} pfu/ml, but gene transfer was significantly less efficient when a concentration of 1×10^{8} pfu/ml was used ($p<0.05$) (Figure 3.3).

In experiment D, viral solution of 1×10^{9} pfu/ml was infused into the aortic root with the pulmonary artery clamped, resulting in the coronary system visibly distended and the virus was then flushed out with cold cardioplegic solution at the end of 60 minutes prior to transplantation. The median (range, SEM) of the total number of positive staining cells for β-galactosidase was 90.2 (25.8 - 118, SEM 13.94). In experiment E, to evaluate

the effect of warm dwell, viral solution of 1×10^{9} pfu/ml was infused into the aortic root with the pulmonary artery opened, and the virus was not flushed out with cold cardioplegic solution at the end of 60 minutes prior to performing heart transplantation. The median (range) of the total number of positive staining cells for β-galactosidase was 3.8 (1.2 - 93.4, SEM 17.8). The efficiency of gene transfer in group D was significantly greater than group E ($p < 0.05$) (Figure 3.4).

There was no significant difference in the aortic root pressure during viral infusion whether the pulmonary artery was clamped or not. The mean pressure reached was 79 mm Hg. The aortic root pressure dropped rapidly to preinfusion level at the end of viral infusion. However, the coronary system remained visibly more distended during and after the viral infusion in hearts in which the pulmonary artery was clamped.

The cell types which stained positively for β-galactosidase included cardiomyocytes, endothelial cells and fibroblasts (Figures 3.5). The smooth muscle cells in the coronary arteries did not stain positive for β-galactosidase. Transduction of cardiomyocytes appeared to be the most efficient of all the cell types. There was no consistent pattern of distribution of staining with the exception that staining appeared somewhat more accentuated around subepicardial zones of organising ischaemia. There was no difference in the extent of transgene expression through both right and left ventricles. All sham sections stained negatively including areas of ischaemic damage. There were no

inflammatory cells seen in either sham or β-galactosidase transduced transplanted hearts.

Discussion

This series of experiments was designed to determine the conditions for optimizing gene transfer in the heart transplant setting. We sought to perform experiments with conditions relevant to clinical transplantation. In agreement with an earlier study using a mouse model [68], the results showed that gene transfer to the transplanted heart using adenoviral vectors is feasible. The efficiency of gene transfer, however, was markedly affected by the experimental conditions.

In clinical heart transplantation, there is up to approximately four hours delay from the time of organ retrieval to transplantation when the donor heart can be safely preserved [83]. Although this may allow for a prolonged viral dwell time for gene transfer, the conditions of organ preservation may have a marked effect on the efficiency of gene transfer. A previous study showed that adenovirus vectors could efficiently transduce hepatocytes *in vitro* at 4^0C but the efficacy was affected by the preservation solution used [84].

In the present experiments, the whole organ was the target for gene transfer. In the initial dose response experiments, gene transfer at 4^0C,

although dependent on vector dose, was inefficient. This level of gene transfer may well be inadequate for effective clinical gene therapy. The reason for the inefficiency was unclear. Adenoviral uptake into cells occurs via receptor-mediated endocytosis [58] and the rate of incorporation into the target cells is temperature dependent [85]. Recently, the steps involved in the attachment and internalisation of the adenovirus in cells were defined [86-88]. The expressions of the CAR (coxsackie and adenovirus receptor) and $\alpha_v\beta_5$ integrin in the target cells are important for efficient adenovirus-mediated gene transfer [86,88]. In addition, it is also known that anatomical barriers may impose significant limitations on the penetration of adenoviral vectors [89]. The penetration of particles in the size range of adenoviral vectors in the arterial system is dependent on the distending pressure [89]. We therefore evaluated the potential benefits of further distending the coronary system and leaving virus in the donor heart during warm ischaemia by clamping the pulmonary artery during viral infusion and not flushing out the viral solution prior to transplantation in experiment B. This resulted in a highly significant improvement in the efficiency of gene transfer. It was noted that four transduced hearts did not show any positive cells for β-galactosidase (experiment A, 2 at 1 x 10^7 pfu/ml, 1 at 1 x 10^8pfu/ml and experiment B, 1 at 1 x 10^8pfu/ml). All hearts transduced at higher concentrations of virus showed positive transgene expression with X-Gal staining. Therefore, the reason for failing to achieve gene transfer in a few of the animals was likely to be related to inadequate viral concentrations for the method of gene delivery used. Biological

variability may also have contributed. However, successful gene transfer was consistently achieved with viral concentrations of 1 x 10^9pfu/ml or greater. Therefore, all subsequent studies in this thesis were performed with this concentration of virus.

To further evaluate the effect of further distension of the coronary system during viral infusion and virus dwell in warm ischaemia, experiments D and E were performed. The results in experiments D and E suggested that coronary distension during viral infusion alone played a greater role in the improved efficiency of gene transfer than a period of virus dwell during warm ischaemia. Although the aortic root pressures measured did not differ during viral infusion with the pulmonary artery open or clamped, this did not exclude the possibility of greater distension in the microvascular system in the latter. Hajjar et al[90] showed successful gene transfer to the heart by infusing viral solution into the coronary system with the distal aorta and pulmonary artery clamped as in experiment B. However, he did this in an *in vivo* rat model with the heart beating. He introduced a cannula into the aortic root via the apex of the left ventricule and subsequently injected the adenoviral vector with the distal aorta and pulmonary artery clamped. The viral solution was allowed to dwell for 10 seconds. This method effectively increased the perfusion pressure down the coronary system during viral infusion and also had the benefit of pulsatile flow. Both these factors may contribute to improved gene transfer efficiency. However, in the setting of clinical transplantation, myocardial preservation is paramount. With this *in vivo* method of gene transfer, the left

ventricular apical puncture and the distension of the ventricles during isovolumetric contractions may significantly add to the myocardial insult already sustained by the donor heart during prolonged cold ischaemic preservation. Donahue et al[91] demonstrated that efficiency of adenoviral-mediated gene transfer to the heart by intracoronary infusion of the viral vector was dependent on the delivery flow rate. The authors hypothesised that flow above a critical rate opened precapillary sphincters and allowed enhanced perfusion of myocardial capillaries, thus maximising the surface area of virus delivery and reducing diffusion distances.

Having identified that the conditions in experiment B resulted in efficient gene transfer, the second dose response experiment showed that a viral concentration of 1×10^{9} pfu/ml remained the most effective dose. The reason for this is unclear but it appears that there is a threshold concentration before efficient gene transfer occurs. In addition, increasing the viral concentration by 10 fold to 1×10^{10} pfu/ml did not increase the efficiency significantly. Higher concentrations of viruses were not used due to the large amount of virus required and also to avoid the potential for cytotoxicity.

To further illustrate the importance of optimising conditions for gene transfer, Donahue et al[92] recently attempted to accelerate and improve the efficiency of gene transfer to *ex vivo* rabbit hearts by manipulating the solution for adenoviral coronary infusion. He exploited the fact that agents like serotonin and bradykinin were known to increase vascular permeability. The integrity of endothelial tight junctions which are regulated by cadherins and

dependent on calcium concentration was also exploited by maintaining a low concentration of calcium in the viral solution. A combination of serotonin and low calcium solution resulted in highly effective and rapid gene transfer.

Several methods of gene transfer to the heart have been studied including direct myocardial injection [62,93] and intracoronary infusion [68,94]. The effectiveness of direct injection was limited by the spatial expression and also the consistent intense inflammatory response seen in the needle track [93]. The present study demonstrated that intracoronary viral infusion resulted in widespread distribution of gene expression and the efficiency of gene transfer was enhanced by coronary distension and a period of warm ischaemia. No inflammatory cells were seen in either sham or β-galactosidase transduced transplanted hearts. This was similarly reported in other studies[61,68]. All histological sections were examined by an experienced pathologist. However, as the hearts were only examined 4 days after transduction, the presence of inflammatory responses could not be excluded if the hearts were examined at other time-points. Therefore, under the conditions of the experiments, there was no evidence of adenoviral-mediated cytotoxicity.

There were areas of ischaemic damage predominantly in the epicardial region. This was most likely due to the inevitable rewarming of the heart during the transplant procedure and the epicardium was most vulnerable due to the intense heat from the operating lights. Although the accentuation of positive staining in zones of organising ischaemia suggested the relevance of warm ischaemia to gene transfer efficiency this was not supported by the

findings in experiment E. The reason for this remained unclear. Recently, Wright et al[95] showed artefactual β-galactosidase staining in necrotic myocardium of rabbit hearts transduced using cationic liposomes or infarcted using coil embolization. Consistently, inflammatory cells were seen in these areas of necrotic myocardium. These cells are known to have high level of endogenous β-galactosidase[96] and the authors suggested this as a possible explanation. However the species, vector, mechanism of infarction and method of staining were different from the present experiments. The level of endogenous β-galactosidase is species-dependent[63]and this may be another factor. In the present experiments, all sham sections (as control) stained negatively for β-galactosidase including areas of ischaemic damage suggesting that artefactual staining was not a problem. However, a cautionary approach to interpreting β-galactosidase staining results should be adopted in view of these potential pitfalls.

Although it appeared as though the efficiency of gene transfer was greater in cardiomyocytes compared to endothelial cells, there were many more cardiomyocytes per cross section of myocardium. We did not determine efficiency of gene transfer by cell type, but similar observations have been made in other animal models [68]. If it is true that gene transfer is greatest in cardiomyocytes, the reason is unclear, but may be related to differential expression of CAR receptors in the different cell types. There was also considerable inter-animal variability in the expression of the transgene. This

may be due to the technique of gene delivery or a reflection of the biological variability of gene transfer. Other studies[63,97] have also reported considerable variability in transgene expression. This variability may compromise clinical gene therapy.

Finally from a biosafety standpoint, although conditions in experiment B were effective in gene transfer, the prolongation of viral dwell time into the period of warm ischaemia and not subsequently flushed out raises concerns related to the subsequent distribution of the virus to other tissues when blood circulation is restored. This will need to be investigated further.

In summary, we have demonstrated a method of adenoviral administration and defined conditions for effective gene transfer to the transplanted rat heart. This method of delivery was used in all subsequent experiments.

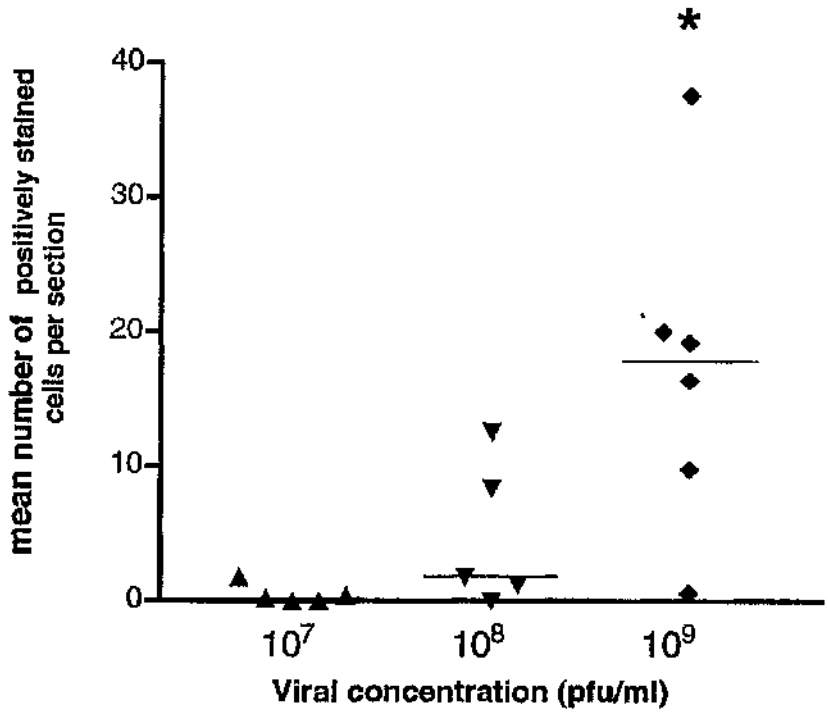

Figure 3.1: Experiment A; A dose response study

Effect of viral dose on the efficiency of gene transfer in the transplanted heart in a syngeneic rat abdominal heart transplantation model under conditions of experiment A, as defined in the text. Data represents median (range) of the number of positively stained cells for β-galactosidase per section. Asterisk (*) denotes significant difference compared to 1 x 10^7 pfu/ml dose (Kruskal-Wallis, Dunn post hoc, $p < 0.05$), n = 5 for groups 1 x 10^7 pfu/ml and 1 x 10^8 pfu/ml, n= 6 for group 1 x 10^9 pfu/ml.

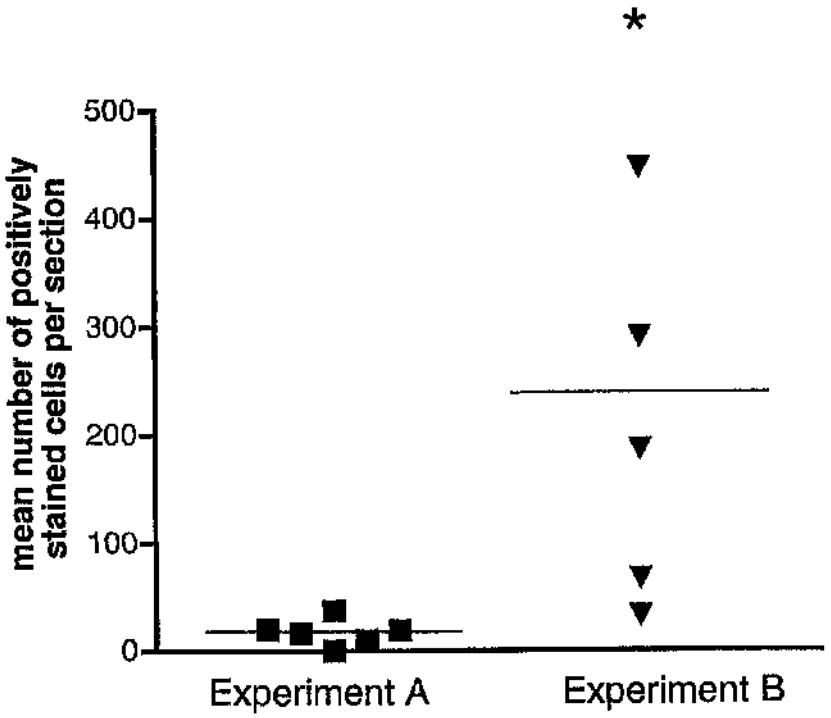

Figure 3.2: Experiment A vs B

Effect of different experimental conditions (experiment A and B), as defined in text, on the efficiency of gene transfer in the transplanted heart. Viral dose of 1×10^9 pfu/ml was used and n = 6 in each group. ---- denotes median value. Asterisk (*) denotes significant difference compared to experiment A (Mann-Whitney test, $p < 0.05$).

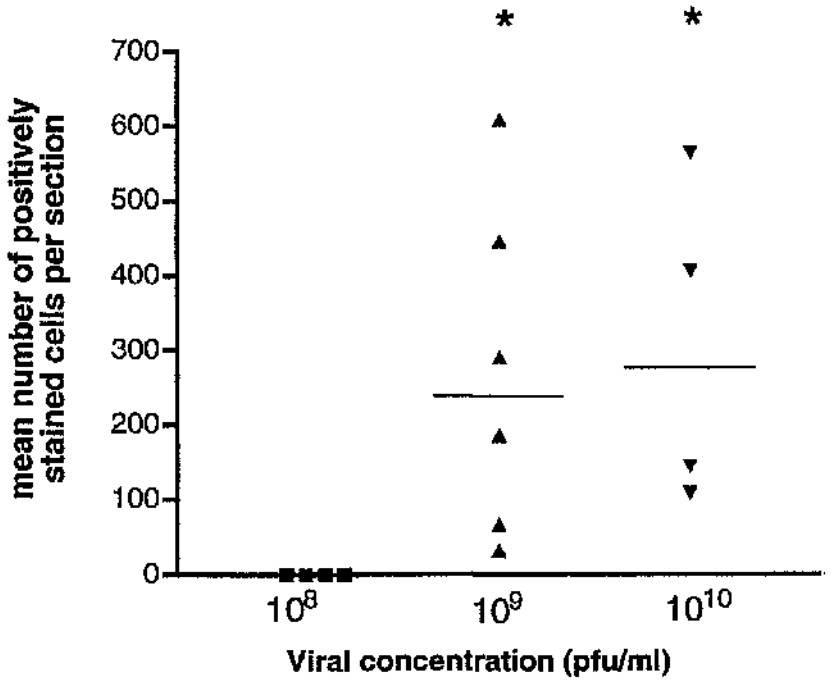

Figure 3.3: Experiment C

Effect of viral dose on the efficiency of gene transfer in the transplanted heart in a syngeneic rat abdominal heart transplantation model under conditions of experiment C, as defined in the text. Data represents median (range) of the number of positively stained cells for β-galactosidase per section. ---- denotes median value. Asterisk (*) denotes significant difference compared to 1 x 10^8 pfu/ml dose (Kruskal-Wallis, Dunn post hoc, $p < 0.05$), n = 4 for groups 1 x 10^8 pfu/ml and 1 x 10^{10} pfu/ml, n= 6 for group 1 x 10^9 pfu/ml.

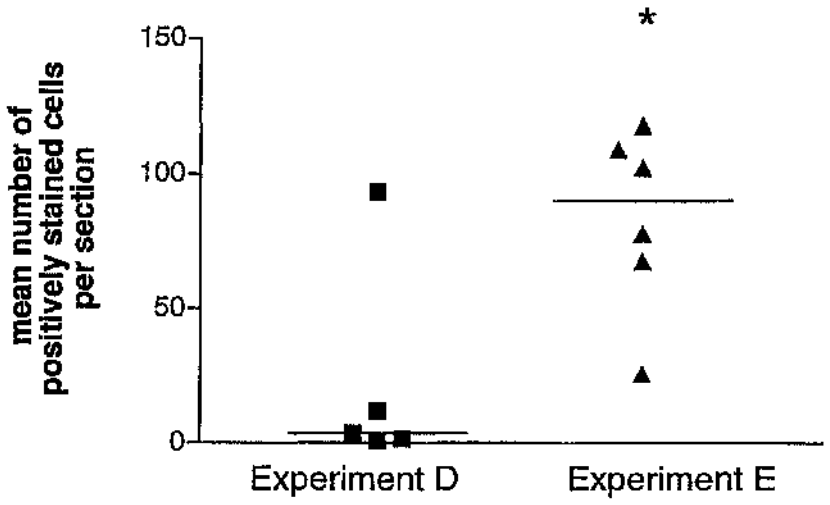

Figure 3.4: Experiment D vs E

Efficiency of gene transfer under conditions in experiment D compared to E as defined in the text. The conditions in experiment D resulted in significantly greater level of transgene expression than experiment E (Mann-Whitney test, $p < 0.05$)

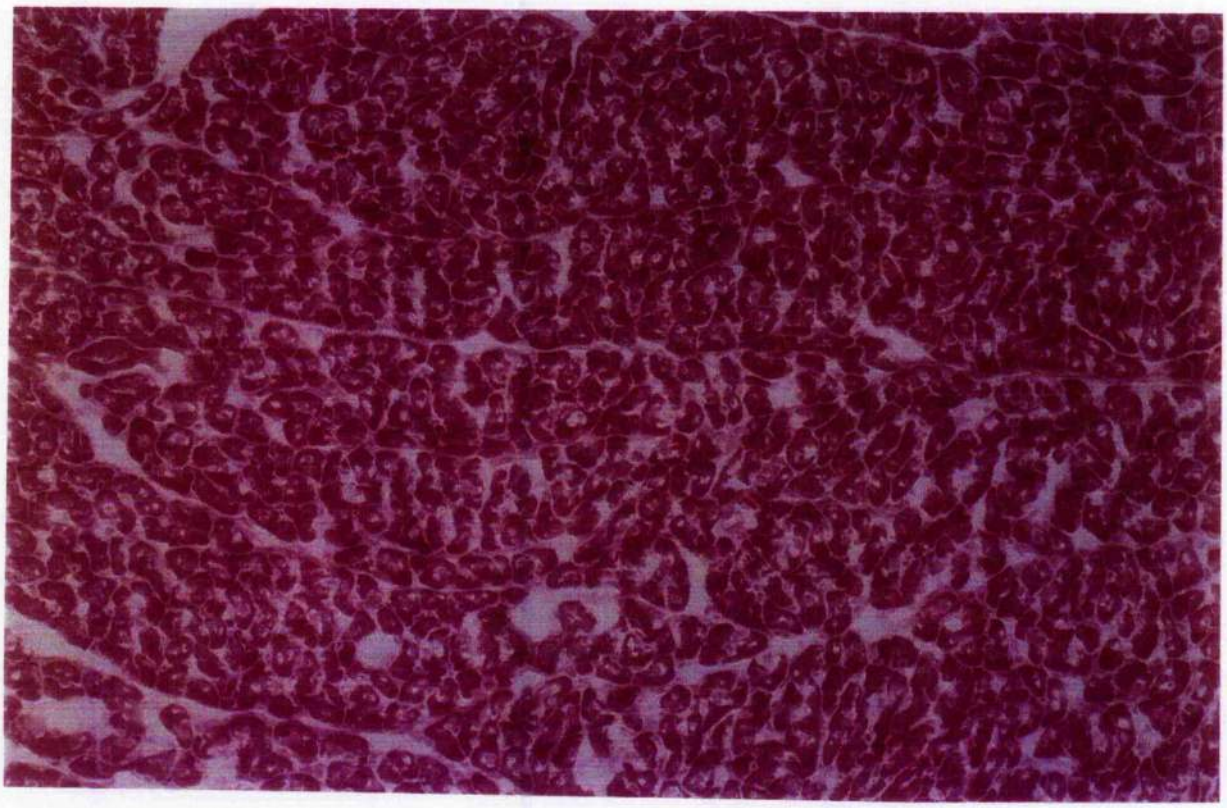

Figure 3.5: X Gal staining of control heart

Myocardium from control animal (experiment A), showing no blue staining, indicating a lack of adenoviral mediated gene transfer of the β-galactosidase gene. x 200

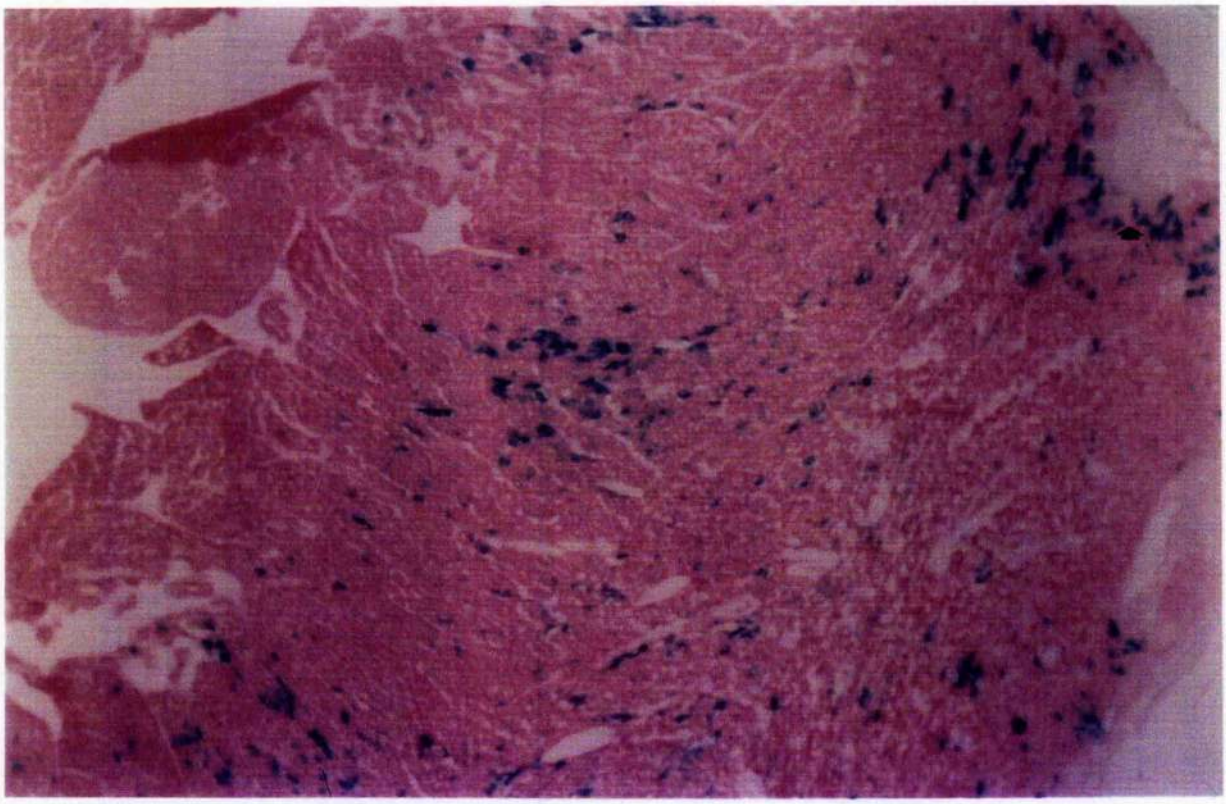

Figure 3.6: Distribution of transgene expression

Myocardium from animal in experiment B showing overall staining intensity and distribution of expression of the β-galactosidase gene as indicated by the blue stained cells. Note positive cells in area of organising ischaemia (arrow) x 100

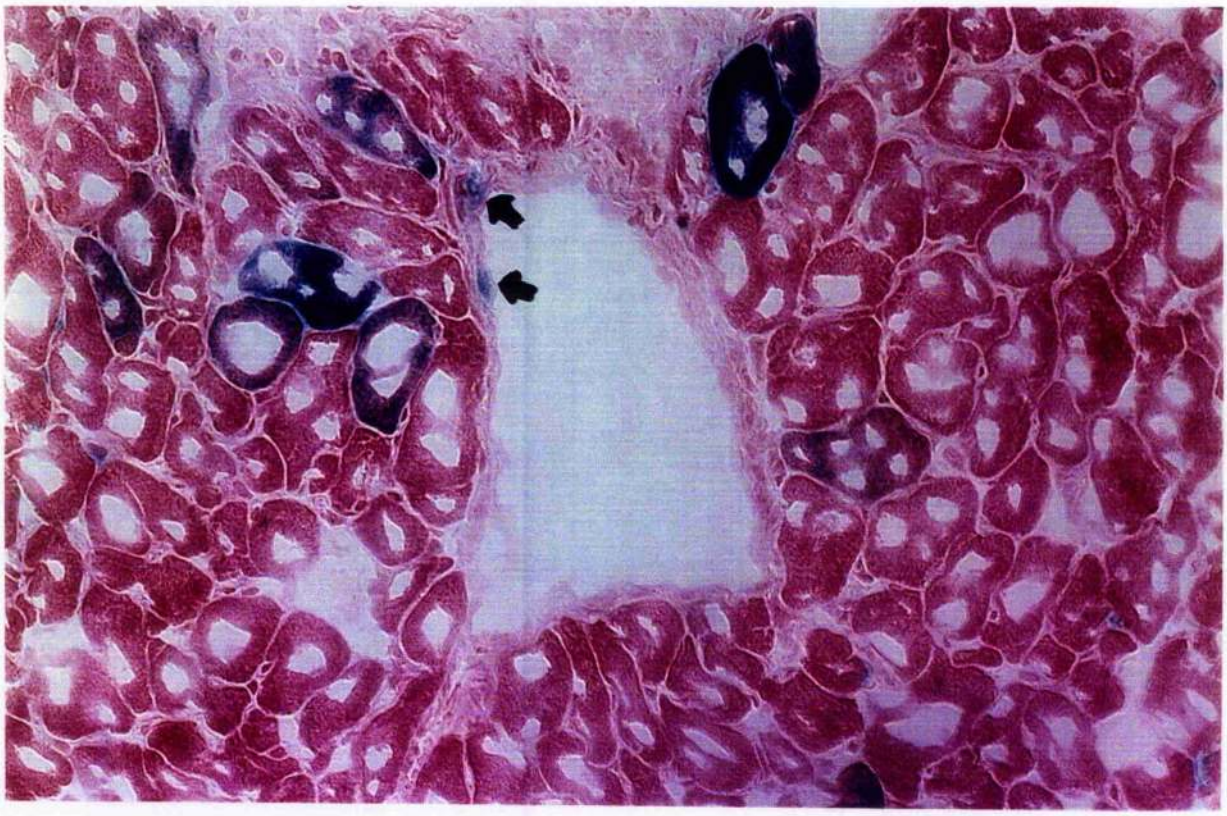

Figure 3.7: Gene transfer to cardiomyocytes and endothelial cells

Myocardium from animal in experiment B showing transduction of cardiomyocytes and endothelial cells. x 400.

Chapter 4 : Influence of Temperature on *In Vitro* Adenoviral-mediated Gene Transfer

Introduction

A significant obstacle to gene therapy is the difficulty of transferring genes to cells and tissues *in vivo*. In contrast to *in vivo* vascular gene transfer, transplantation may represent an ideal setting for administration of the vector via the blood vessels as the inevitable period of donor organ ischaemia following harvesting allows a prolonged dwell time within the target tissue. In the previous chapter, the efficiency of gene transfer was markedly affected by conditions of vector delivery. The efficiency of gene transfer during the period of cold ischaemia may be influenced by the low temperature of the preservation solutions. The endothelial surface represents the initial barrier to gene transfer in the heart. This study was designed to examine the effect of temperature on the efficiency of adenovirus-mediated gene transfer to endothelial cells *in vitro*. As this is an *in vitro* cell culture study, the conditions for gene transfer would be different from that to an intact organ. Therefore extrapolating the results to an *ex vivo* or *in vivo* gene transfer to an intact organ setting should be done with caution.

Methods

Adenovirus Vector

A replication defective, E1a-deleted serotype 5 adenoviral vector

encoding for β-galactosidase, under the control of the CMV promoter was used in this study. The propagation and purification of the vector have been previously described in Chapter 2.

Transduction of cells *in vitro*

Endothelial cells were purchased from Clonetics Corporation (San Diego, CA). They were grown in endothelial cell basal medium without phenol red (Clonetics Corporation, San Diego, CA) supplemented with human recombinant epidermal growth factor (0.01 ng/ml), hydrocortisone (1 μg/ml), gentamicin (50 μg/ml), amphotericin B (0.05 μg/ml), bovine brain extract (12 μg/ml) and 2 % fetal bovine serum.

Endothelial cells were incubated in a humidified, 5% carbon dioxide atmosphere at 37° C and routinely passaged just before reaching confluence by brief exposure to trypsin-EDTA solution (trypsin 0.05%, EDTA 0.53 mM; Gibco BRL, Gaithersburg, MD). Cells at passage 4 - 8 were used in all experiments. 5×10^4 cells were plated on 6-well plates and allowed to attach overnight.

After removal of medium, cells were washed twice with phosphate-buffered saline (Gibco BRL, Gaithersburg, MD) and then incubated for 1 hour in 0.5 ml of phosphate-buffered saline + 0.1 % bovine serum albumin containing 2×10^7 pfu/ml, which was equivalent to a multiplicity of infection (MOI) of 200. MOI is equivalent to the number of viral particles per cell.

Endothelial cells were incubated at three different temperatures: 4°C, 10°C and 37°C. At the end of incubation period the cells were washed twice with phosphate-buffered saline and incubated for 24 hours in fresh medium. In all experiments (n = 4), 12 wells for each cell type at each incubation temperature were used. Control cells were incubated using the same experimental conditions in virus-free phosphate-buffered saline + 0.1 % bovine serum albumin.

Twenty four hours later cell viability was assessed by trypan blue exclusion. The viability of two wells was evaluated using a standard haemocytometer following detachment of cells by trypsin-EDTA solution.

Prior to staining for β-galactosidase, endothelial cells were washed twice with phosphate-buffered saline and fixed for 15 minutes in 1.25% glutaraldehyde at 4° C. After a further rinse in phosphate-buffered saline, the cells were stained in a solution of 500 μg/ml X-Gal for 4.5 hours at 37° C. Blue stained cells indicated the presence of β-galactosidase expression. For quantitative analysis, the total number of positively staining and negative cells were counted manually by an observer blinded to experimental groups using an inverted bright light microscope (magnification = 200x). Fourteen randomly selected fields in each well were evaluated. The percentage of transduced cells was calculated as the number of positively staining cells divided by the total number of counted cells x 100.

Statistical analysis

Results were expressed as mean ± standard deviation. The characteristic of the data suggested equal variances. The analysis of variance (parametric) was performed to evaluate overall differences between the three groups. If overall significance was present, Bonferroni's post hoc test was used for pair comparisons (Prism, GraphPad, San Diego). A P value of less than 0.05 was considered significant.

Results

In vitro Gene Transfer to Cells

Toxicity: the viability assessed at the end of the 24 hours incubation ranged from 75 to 80 % in endothelial cells transduced at 4° C and 10° C and from 80 to 85 % in cells transduced at 37° C.

Human umbilical vein endothelial cells: following transduction at 4° C and 10° C, the percentage of cells positively stained for β-galactosidase was 14.29 ± 3.79 % and 12.43 ± 2.47 %, respectively. No significant difference was noted between these groups. In contrast, when the cells were transduced at 37° C, the positively stained cells were 30.55 ± 7.26 % ($p < 0.01$ vs. 4° C and

10° C) (Figure 4.1). Control endothelial cells, not exposed to the adenoviral vector, stained negative for β-galactosidase.

Discussion

As donor organs are preserved at 4° C, the effect of temperature on gene transfer is an important question. The present study demonstrates that efficiency of adenoviral-mediated gene transfer to endothelial cells *in vitro* is significantly influenced by temperature. This may represent an impediment to adenoviral-mediated gene therapy in the setting of clinical transplantation as during retrieval, the donor organ is perfused and stored in cold preservation solution to preserve cellular viability until the transplant procedure can be accomplished. Although transplantation may be an ideal setting for gene therapy, as a prolonged dwell of the vector in the target organ can be achieved during this inevitable period of ischaemia, gene transfer may be influenced by the low temperature of cold preservation.. In the previous chapter, it was noted that transgene expression was accentuated in superficial and less well preserved areas of the heart. This observation suggested the role of temperature on the efficiency of gene transfer as the subepicardial layer of the heart rewarmed quicker, during the transplant procedure, due to its proximity to the operating light source.

The effect of temperature on the gene transfer has not been fully addressed. In a study by Csete et al, gene transfer to the donor liver was not affected by low temperature[84]. After 1 hour exposure to the adenoviral vector at 4° C, efficient transduction was noted (about 80 % of hepatocytes showed positive staining for the reporter gene). However, this high efficiency may be related to the marked tropism of this vector for hepatocytes. In another report, adenoviral-mediated gene transfer to endothelial cells *in vitro*, in organ cultures and *in vivo* was influenced by temperature[98]. In that study, efficiency of gene transfer to vascular endothelial cells *in vitro* appeared to be dependent on the titre of the viral solution, whereas the endothelium *in situ* seemed to be resistant to adenoviral infection even at the highest viral titre (10^{11} pfu/ml). The latter finding has not been observed by others. In contrast, Chapelier et al. [99] have demonstrated that efficiency of adenoviral gene transfer to endothelial cells was significantly impaired at 10° C. The results from the present study are consistent with those of Chapelier. Recently, Donahue et al[92] proposed altering the conditions for adenoviral gene transfer by using a viral solution which was low in calcium and had serotonin added. The effect of both these components theoretically increases the permeability of the endothelial barrier to gene transfer. Further techniques of optimising conditions for gene transfer were discussed in the previous chapter.

In clinical transplantation, efficient gene transfer to the donor organ will be necessary at 4° C. If inefficient, gene transfer in this setting results in

generation of low quantities of biologically active transgene, efforts will be needed to improve gene transfer efficiency at this temperature. Although increasing the dose of the vector may enhance gene transfer efficiency, this may also augment the risk of vector-induced toxicity. As efficiency of adenoviral gene transfer is directly related to the length of exposure to the vector[69], improvement of donor organ preservation may allow more prolonged dwell time within the target organ and thus may afford enhanced gene transfer efficiency.

Finally, there are clear differences between the environmental conditions of *in vitro* cell culture and intact organ. The main advantage of using an *in vitro* cell culture technique for testing the role of temperature in adenoviral gene transfer is the ability to control the environment precisely. In addition, the cells of *in vitro* culture may be exposed directly to the adenoviral vector and gene transfer may be achieved at a lower and defined concentration. One of the disadvantage of cell culture environment includes the loss of specific cell interactions which are characteristics of intact organs. This may significantly alter the efficiency of gene transfer in an intact organ. In addition, it also lacks the systemic components that may influence the efficiency of gene transfer in an intact organ *in vivo*. Therefore , as long as the limitations of the *in vitro* cell culture technique are appreciated, it can be a valuable tool for studying gene transfer.

In summary, this study demonstrates that efficiency of adenovirus-mediated gene transfer to endothelial cells is significantly reduced by low

temperature. Requirement of donor organ cold preservation may undermine the effectiveness of gene therapy in the transplant setting.

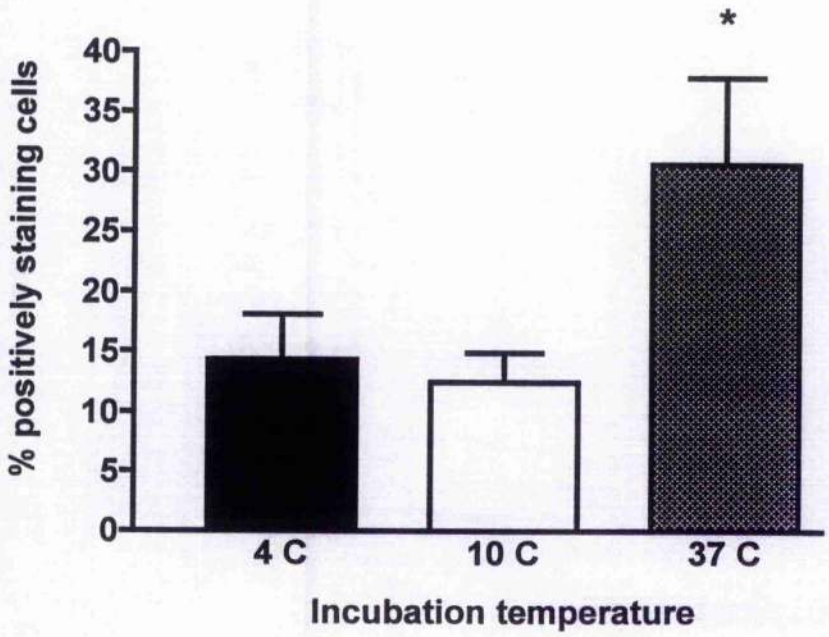

Figure 4.1: Effect of temperature on *in vitro* gene transfer

Effect of incubation temperature on gene transfer efficiency to human umbilical vein endothelial cells (HUVEC). Multiplicity of infection = 200. Data represent mean ± SD of the percentage of cells positively staining for the reporter gene (wells no. = 10, experiment performed on 4 separate occasions). Asterisks denote significant difference compared to cells at 4° C and 10° C (ANOVA, Bonferroni's post hoc test, $p < 0.05$)

Chapter 5 : Immunosuppression Prolongs Adenoviral-Mediated Transgene Expression in Cardiac Allograft Transplantation.

Introduction

Chapter 1 demonstrated the feasibility and efficiency of adenoviral-mediated gene transfer in the heterotopic heart transplantation model. However, one major limitation of first generation adenoviral vectors has been the transient nature of transgene expression. In previous studies using adenoviral vectors, transgene expression was lost or greatly diminished after 30 days [68,93]. Although the reason for this is not entirely clear, cell mediated immune responses appear to play an important role in the initial loss of transgene expression and the development of neutralising antibodies by activated B cells reduces the effectiveness of subsequent adenoviral transduction [71,73]. The prolongation of transgene expression in transduced immunologically immature neonatal animal models [57]and with the use of immunosuppression [74]further supports the role of the immune system in this process.

In the heart transplantation setting, the necessary use of immunosuppression and the possibility of a prolonged viral dwell time during the period of donor organ ischaemia may be a more ideal setting for efficient gene transfer and prolonged transgene expression. In addition, readministration of the adenoviral vector may not be applicable in this setting as direct access to the heart may be impractical. This study therefore hypothesises that in the

setting of heart transplantation, the routine use of immunosuppression will prolong transgene expression in adenoviral-mediated gene transfer.

Methods

Adenovirus Vector

A replication defective adenovirus vector encoding for β-galactosidase under the control of the CMV promoter was used as reporter gene. The recombinant virus was propagated in 293 cells and then isolated and purified as described in Chapter 2.

Animals

A total of 152 rats (donors and recipients) were used in these experiments. Inbred male Lewis (RT-1^{l}) rats were used as syngeneic donors and Brown Norway (RT-1^{n}) rats were used as allogeneic donors. All recipients were Lewis rats (RT-1^{l}).

Operation and Gene Transfer

Rat heterotopic abdominal heart transplantations, using standard microsurgical techniques as described in Chapter 2, were performed.

The donor hearts were transduced with adenoviral vectors under conditions defined in Chapter 2 that resulted in the most efficient gene transfer (in experiment B). Briefly, a volume of 350µl of viral solution at a concentration of 1×10^{9} pfu/ml was infused over 5 seconds into the coronary arteries via the aortic root. The pulmonary artery was clamped during viral infusion and the virus was not flushed out at the end of 60 minutes cold storage prior to performing heart transplantation. All donor hearts were heterotopically transplanted into the abdomen of the recipients. The total period of incubation with the virus was 90 minutes with 60 minutes cold ischaemia during the cold storage period plus a further 30 minutes of warm ischaemia during the transplant procedure when inevitable rewarming of the heart occurs. Function of the grafts was checked daily by palpation of the beating transplanted heart.

Experimental Groups

The duration of transgene expression was examined following:

a) syngeneic transplantation in non-immunosuppressed animals (**Group A**),

b) syngeneic transplantation in immunosuppressed animals (**Group B**)

c) allogeneic transplantation in immunosuppressed animals (**Group C**).

Immunosuppressed animals were given cyclosporine by daily orogastric injections from the day of surgery. The cyclosporine dose was 10mg/kg/day for the first 2 weeks after transplant and 5mg/kg/day thereafter until harvest [76]. This dosage was chosen as a balance between effective immunosuppression and fatal toxicity of the drug. All animals were weighed weekly for dose adjustment. After transplantation, animals were studied at 1, 4, 8 and 12 weeks (n=6 in each group for each time point giving a total number of 72 transplants). These prolonged time points were chosen to study the effect of immunosuppression on the duration of transgene expression. Previous studies[68,100] had shown that 30 days after adenoviral-mediated gene transfer, transgene expression was lost or greatly diminished. However, the study suggested that immunosuppression may prolong transgene expression but the duration was not investigated.

Heart Excision, Histochemical Analysis and Rejection Staging

At the termination of the experiment, transplanted hearts were removed and flushed with saline. Specimens for analysis were prepared as described in the methods section of Chapter 2. Blue stained cells indicated the presence of β-galactosidase expression. The overall mean values of 5 midventricular sections were calculated for each group and the results expressed as mean and median number of positively stained cells per section were then analysed. For assessment of rejection, formalin preserved heart

sections were embedded in paraffin, cut, stained with haemotoxylin and eosin and then scored according to the guidelines set by the Heart Rejection Working Group [78]. All slides were read by an experienced cardiac pathologist blinded to the identification of the slides.

Statistical analysis

Results were expressed as mean ± standard deviation, and median (range) of the number of positive staining cells per cross section. As the data did not follow a Gaussian distribution and variances were unequal, a non-parametric test (Kruskal-Wallis) of analysis of variance was performed to evaluate overall group differences for more than two groups. If overall significance was present, Dunn's post hoc test was used for pair comparisons (Prism, GraphPad, San Diego, CA). A p value of < 0.05 was considered significant.

Results

Two animals died early, from post-operative bleeding, and at 8 and 12 weeks giving an overall mortality of 5%. All transplanted hearts in the study were beating at the time of harvest.

Group A (Non-immunosuppressed syngeneic): The total number of positively stained cells for β-galactosidase activity was 240.6 ± 171.9 (mean ± standard deviation) with a median range of 254.3 (24-510.6) at 1 week, 0.1 ± 0.1 and 0.1 (0-0.2) at 4 weeks, 0.03 ± 0.08 and 0 (0-0.2) at 8 weeks and 0.7 ± 0.8 and 0.4 (0-2) at 12 weeks. (Kruskal-Wallis, non parametric ANOVA, $p<0.05$ for all time-points comparisons) (Figure 5.1). Four weeks after transduction, transgene expression was virtually undetectable.

Group B (Immunosuppressed syngeneic): The total number of positively stained cells was 374.2 ± 488.2 and 218 (7.4-1307) at 1 week, 142.5 ± 135.2 and 80.5 (18.6-321.8) at 4 weeks, 44.2 ± 48.1 and 31.2 (0.4-121.4) at 8 weeks and 13.7 ± 23.7 and 4.8(0-61.6) at 12 weeks (Kruskal-Wallis, non parametric ANOVA, $p<0.05$ for all time-points comparisons) (Figure 5.2). The use of immunosuppression in these syngeneic transplants resulted in prolongation of transgene expression.

Group C (Immunosuppressed allogeneic): The total number of positively stained cells was 697.1 ± 517.7 and 827 (32.4-1352) at 1 week, 250.8 ± 104.2 and 224.5 (142.6-386.6) at 4 weeks, 162.5 ± 201.4 and 45.2 (0-476.4) at 8 weeks and 75.9 ± 141 and 8.8 (0-326.8) at 12 weeks (Kruskal-Wallis, non parametric ANOVA, $p>0.05$ for all time-points comparisons) (Figure 5.3). As with group B, immunosuppression in allogeneic transplants resulted in prolongation of transgene expression.

In group A (non-immunosuppressed syngeneic group), transgene expression was virtually undetectable by 4 weeks, whereas in both groups B (the immunosuppressed syngeneic) and C (immunosuppressed allogeneic), expression of the reporter gene persisted until the termination of the experiment at 12 weeks. However, the number of positive staining cells per section in both groups decreased substantially with time. There were no significant differences in the number of positive staining cells between the three groups at 1 week although there was a trend towards more positively stained cells in group C (Figure 5.4a). At 4 weeks, transgene expression in group C (immunosuppressed allogeneic) was significantly greater than group A (non-immunosuppressed syngeneic), (Figure 5.4b) but not group B (immunosuppressed syngeneic). At 8 weeks, transgene expression in both group B (immunosuppressed syngeneic) and C (immunosuppressed allogeneic) was significantly greater than group A (non-immunosuppressed syngeneic), (Figure 5.4c). At 12 weeks, there were no significant differences among the three groups (Figure 5.4d).

Despite the use of immunosuppression, histological evidence of rejection was present in many of the allogeneic animals. At 1 week, 4 animals were completely free of rejection while 2 had evidence of moderate grade rejection (3A). At 4 weeks, 1 animal had low grade (1A) and 5 had moderate grade (3A) rejection. At 8 weeks, 2 animals had low grade (1A) and 4 had moderate grade (3A) rejection. At 12 weeks, 3 animals had no rejection and 3 had low grade (1A to 2) rejection. In the non-immunosuppressed syngeneic

group, 2 animals at 4 weeks, 1 animal at 8 weeks and 2 animals at 12 weeks had patchy mononuclear cell infiltrates with rare foci of myocyte damage.

Consistent with the findings in Chapter 3, the cell types that stained positively for β–galactosidase included cardiomyocytes, endothelial cells and fibroblasts. Smooth muscle cells in the coronary arteries did not stain positive for β–galactosidase. As in earlier experiments, the cardiomyocyte was the predominant transduced cell type. The cell types were determined on the basis of morphology by the experienced pathologist. There was no consistent pattern of distribution of staining with the exception that staining appeared somewhat more accentuated in the subepicardial region.

Discussion

Adenoviral vectors can achieve efficient gene transfer in different animal models and in a variety of cell types including non-dividing cells. The advantages of these vectors have been previously outlined [81]. One of the major limitations of these vectors has been the transient nature of transgene expression. Previous studies using adenoviral vectors have shown that transgene expression is lost or greatly diminished after 30 days [68,93]. This may render these vectors unsuitable for gene therapy approaches to chronic disorders. However, there may be situations when transient gene expression is sufficient to modify the pathological processes. It is also possible that

immunosuppressive agents may prolong transgene expression[74,100]. This may be of particular relevance in situations, such as heart transplantation, where these agents are routinely administered.

This study has demonstrated for the first time that immunosuppression prolongs transgene expression in the heart transplant setting. Transgene expression was significantly greater at 8 weeks in the immunosuppressed syngeneic and allogeneic animals than in the non-immunosuppressed animals. At 12 weeks, although there was no statistically significant difference in the number of positive staining cells per section detected between the 3 groups, transgene expression was still detectable in the immunosuppressed groups.

The limited duration of transgene expression after adenoviral-mediated gene transfer may be due to immune-mediated mechanisms [71]. In support of immune mechanism playing a role, prolonged transgene expression has been reported in immunologically immature neonatal animals [57], in immunodeficient animals [101] and with the use of immunosuppression [74]. Furthermore, while some authors report the absence of inflammatory infiltrates in the myocardium after catheter-mediated delivery of adenoviral vectors to both transplanted and non-transplanted hearts[61,68], direct injection has been associated with inflammation around the injection site[62,97,102]. While some of the inflammation may be due to the effect of the needle, immune responses to the viral vectors may also be responsible. The latter possibility is supported by the results of this study. In this study, transgene expression was barely detectable in the

non-immunosuppressed syngeneic hearts by 4 weeks; but patchy lymphocytic infiltrates consistent with myocarditis were noted in 2 of 6 animals at 4 weeks, 1 of 6 animals at 8 weeks and 2 of 6 animals at 12 weeks. These lymphocytic cell types were determined on the basis of cellular morphology from H & E staining. This may indicate an immune response to the vector or transgene even in the absence of detectable transgene expression in these animals. Additionally, this study demonstrated that transgene expression can be prolonged by immunosuppression with cyclosporine after heart transplantation further implying an important role for immunological factors . It was noted that there was considerable variability in the number of positive staining cells per section in each group of animals. This topographical variability in cellular transduction efficiency may be due to unexplained biological variability of adenoviral-mediated transgene expression. It may also be a result of the method used in vector delivery in these experiments.

While transgene expression was prolonged to at least 8 weeks with the use of cyclosporine, in both the syngeneic and allogeneic immunosuppressed groups, there was a significant decline with time despite immunosuppression. This may be due to suboptimal dosage of cyclosporine used in the current study or non-immune mechanism limiting persistent transgene expression. In support of the former hypothesis, rejection was frequently detected by histological examination in the allogeneic animals suggesting that the dose of immunosuppression used in this study was insufficient to prevent rejection. However, there was no correlation between the number of positive staining

cells and cellular infiltrate or degree of rejection in any of the 3 groups. In a recent study, similar decrease in transgene expression was observed in myocardium of athymic rats transduced with an adenoviral vector [101] suggesting that non-immune mediated mechanism may also limit the duration of transgene expression.

In addition to manipulating the immune response to adenoviral vectors, as was done in this study, the problem of transient transgene expression is also being addressed by modifications in vector design. Second generation adenoviral vectors which have an E1a deletion and a temperature sensitive mutation in the E2a region have been shown to diminish viral protein expression [103]. In a mouse model[104], this has been shown to prolong transgene expression in the liver to at least 8 weeks. Recombinant adenoviral vectors with all viral genes deleted and which required the use of helper virus for propagation have also been generated, but separation of recombinant and helper virus remained a problem with the use of these vectors[105]. Further refinement in vector generation may ultimately lead to a vector system that is efficient, non toxic and capable of long term transgene expression.

In conclusion, this study demonstrates that transgene expression using adenoviral vectors is prolonged by immunosuppression in the heart transplantation setting. The decrease of transgene expression with time is probably due to a combination of immune and non-immune mechanisms. This study suggests that the routine use of immunosuppression in heart transplant setting may prolong transgene expression but the level of expression decreases

significantly with time. The effect of alternative immunosuppressive regimens on transgene expression in the heart transplant setting is worthy of further study.

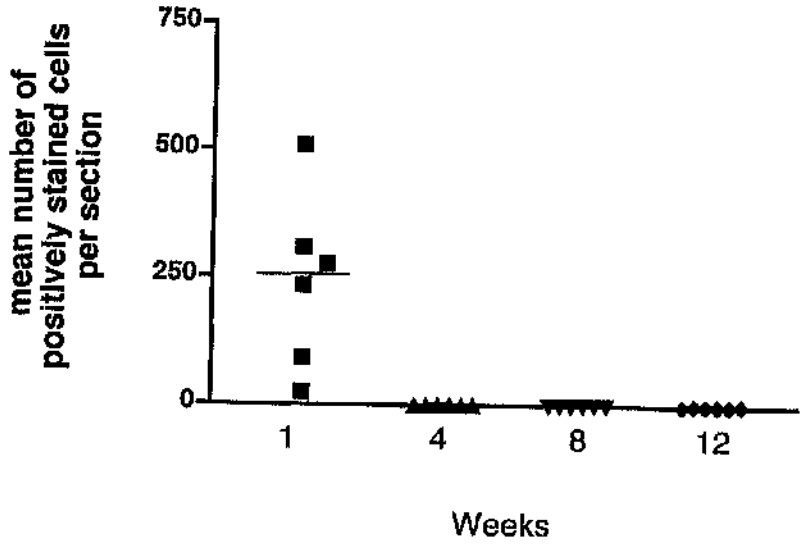

Figure 5.1: Duration of transgene expression in non-immunosuppressed syngeneic heart transplants (Group A)

Data represents mean number of positively stained cells for β-galactosidase per section for each animal . — denotes median value. Viral concentration of 1 x 10^9 pfu/ml was used in all experiments, n = 6 for each time-point. The level of transgene expression dropped significantly after week 1 (Kruskal-Wallis, non parametric ANOVA, $p<0.05$).

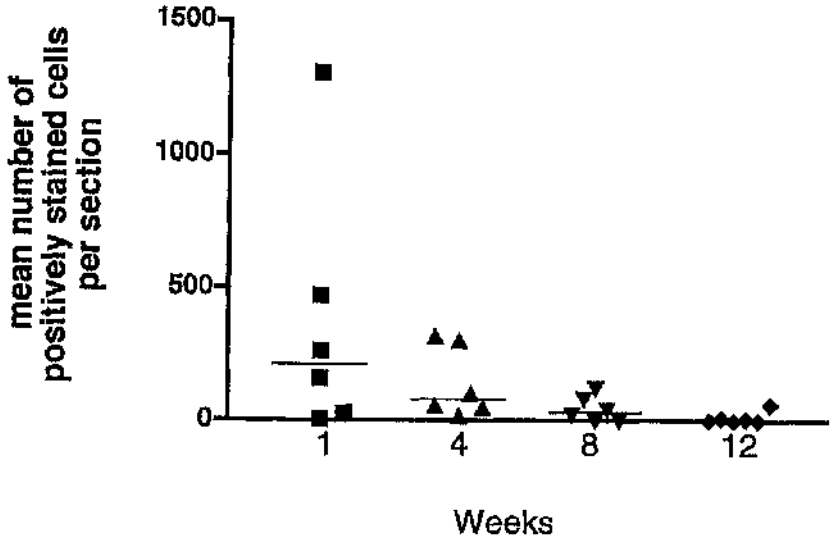

Figure 5.2: Duration of transgene expression in immunosuppressed syngeneic heart transplants (Group B)

Data represents mean number of positively stained cells for β-galactosidase per section for each animal. Viral concentration of 1 x 10^9 pfu/ml was used in all experiments, n = 6 for each time-point. ⎯⎯ denotes median value. (Kruskal-Wallis, non parametric ANOVA, $p<0.05$).

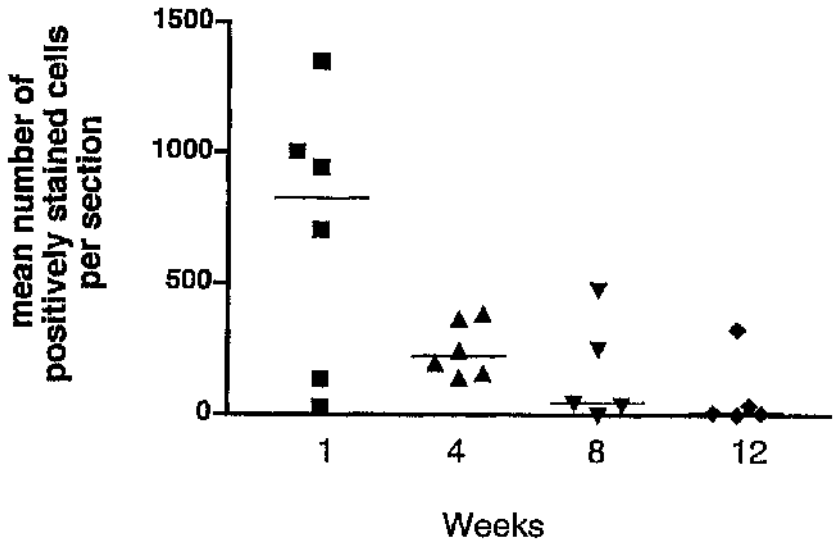

Figure 5.3: Duration of transgene expression in immunosuppressed allogeneic heart transplants (Group C)

Data represents mean number of positively stained cells for β-galactosidase per section for each animal. —— denotes median value. Viral concentration of 1 x 10^9 pfu/ml was used in all experiments, n = 6 for each time-point except week 8 and 12, n = 5. (Kruskal-Wallis, non parametric ANOVA, $p>0.05$).

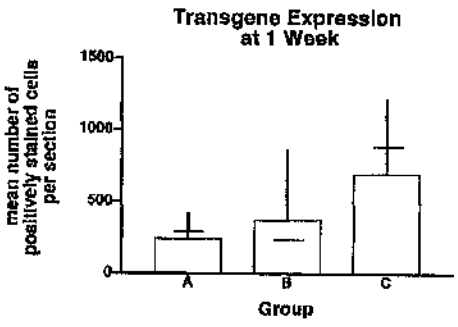

Fig 5.4a

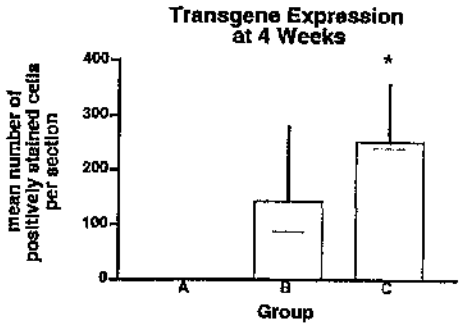

Fig 5.4b

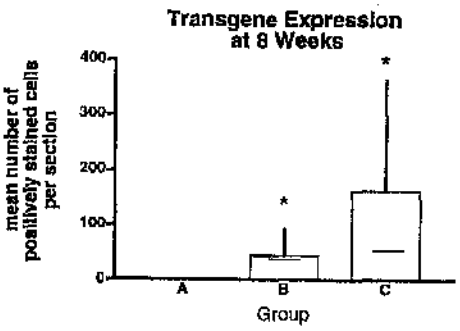

Fig 5.4c

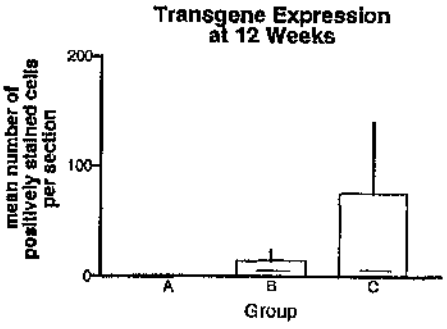

Fig 5.4d

Figure 5.4 a - d: Comparison of level of transgene expression in Group A, B and C at 1, 4, 8 and 12 weeks

Data represents mean ± SD of the number of positively stained cells for β-galactosidase per section. — denotes median value. Asterisk (*) denotes significant differences compared to group A. There was no significant differences between all groups at 1 and 12 weeks (Kruskal-Wallis non parametric ANOVA, Dunn's post hoc, $p>0.05$).

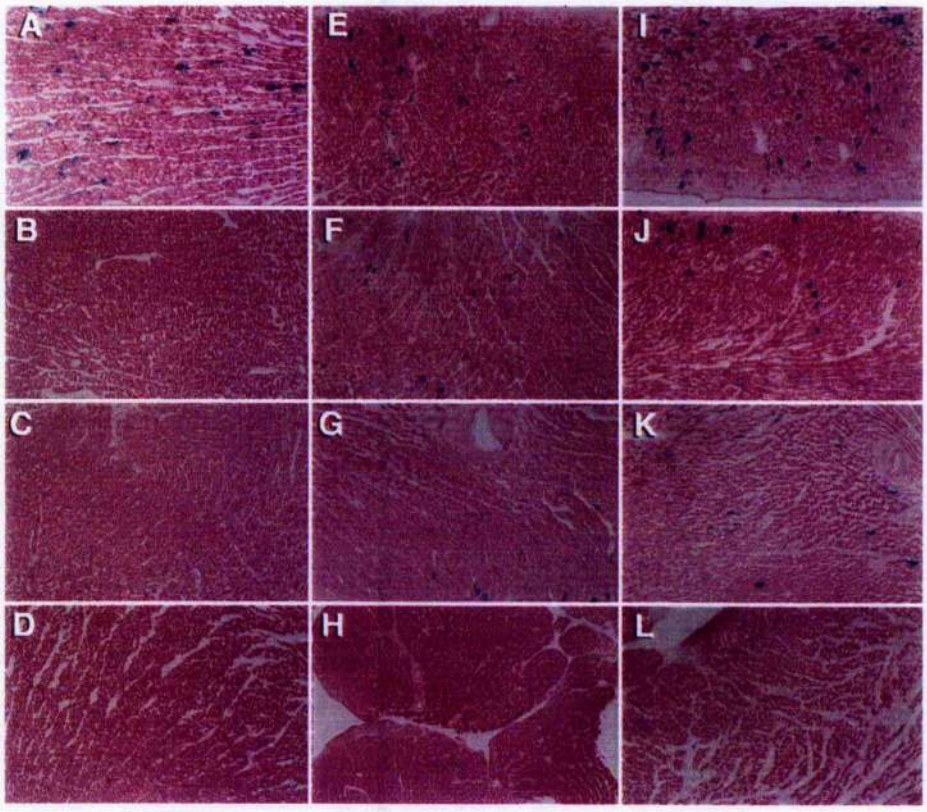

Figure 5.5: Transgene expression by histochemical staining for beta-galactosidase at 1, 4, 8 and 12 weeks in all groups

The blue cells are positively stained. Panels A - D demonstrate β-galactosidase expression in syngeneic non-immunosuppressed animals at 1, 4, 8 and 12 weeks, respectively. Panel E - H demonstrate β-galactosidase expression in syngeneic immunosuppressed animals at 1, 4, 8 and 12 weeks, respectively. Panel I - L demonstrate β-galactosidase expression in allogeneic immunosuppressed animals at 1, 4, 8 and 12 weeks, respectively. Note that the greatest transgene expression is in the immunosuppressed allogeneic animals (panels I - L) and that in all three groups the level of expression decreases with time. (x 100)

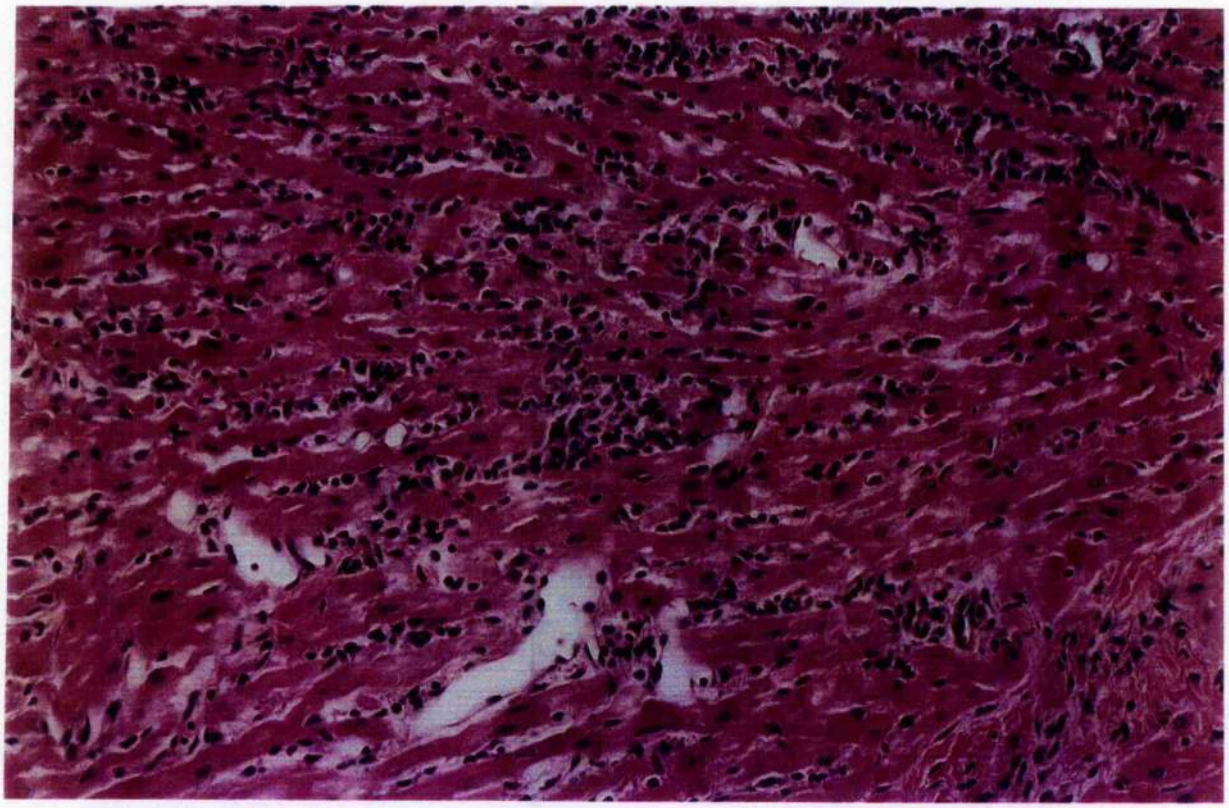

Figure 5.6: Allograft rejection

Evidence of moderate rejection (grade 3A) in allogeneic immunosuppressed heart transplant. (x 100)

Chapter 6 : Distribution and Function of Recombinant Endothelial Nitric Oxide Synthase in Transplanted Hearts

Introduction

Introduction of recombinant genes into donor hearts may offer a therapeutic intervention that could potentially attenuate the complications of heart transplantation including rejection, infection and accelerated transplant atherosclerosis. The aetiology and pathogenesis of accelerated transplant atherosclerosis appears multi-factorial and non-immune mechanisms including early ischaemia-induced endothelial cell injury, ischaemic reperfusion and cytomegalovirus infection may all contribute[13].

In the cardiovascular system, reduced bioactivity of endothelial nitric oxide is a feature of atherosclerosis and vascular injury. Nitric oxide (NO) derived from the vascular endothelium regulates vascular tone and inhibits proliferation of smooth muscle cells, and adhesion and activation of leukocytes and platelets [47,106-109]. Therefore, overexpression of nitric oxide may be beneficial in modifying accelerated heart transplant atherosclerosis. Indeed, overexpression of iNOS (by gene transfer) attenuates intimal hyperplasia in aortic allografts in rats[109]. However, the role of nitric oxide in a solid organ such as the heart is complex. Under physiological conditions, basal release of nitric oxide in the heart is important in preserving ventricular function[110]. In contrast, excessive production of NO, secondary to activation of the inducible form of nitric oxide synthase (iNOS), which occurs in conditions such as allograft rejection[111] and endotoxaemia reduces myocardial contractility[112-114].

The initial experiments in this thesis established the feasibility of adenoviral-mediated gene transfer in the transplanted heart using a reporter gene. In order for adenoviral-mediated gene transfer to be a useful tool for studying the pathogenesis of the processes affecting transplanted hearts, the feasibility of overexpressing biologically relevant and important genes need to be addressed. Therefore, experiments were designed to determine the distribution and functional consequences of overexpressing endothelial nitric oxide synthase (eNOS) gene in the transplanted heart.

Methods

Construction, Propagation and Purification of Adenoviral Vector

A recombinant adenovirus containing the cDNA encoding eNOS was generated as described in Chapter 2. Briefly, bovine eNOS cDNA was cloned into the shuttle plasmid pACCMVpLpA. The resulting plasmid was linearised with NruI and cotransfected with d1309 into 293 cells by calcium phosphate/DNA coprecipitation. d1309 is a biologically selected, restriction enzyme-site-loss variant of wild type adenovirus type 5 which retains only a single XbaI site at nucleotide 1339. 293 cells are human embryonic kidney carcinoma cells which have been transformed with the left end of human adenovirus type 5 DNA. Recombinant adenoviral vectors were generated by

homologous recombination. The adenoviral vector encoding for the reporter gene β–galactosidase (AdLacZ) was used in all experiments as control.

Animals, Operation and Gene Transfer

Inbred male Lewis (RT-1^{l}) rats (250 to 300 grams) were used as donors and recipients.

Heterotopic abdominal heart transplantation using standard microsurgical techniques was performed as previously described. For gene transfer, either adenoviral vector carrying the eNOS or the LacZ (control) gene at a concentration of 1×10^{9} pfu/ml (total volume 350 µl) was infused over 5 seconds into the coronary arteries via the aortic root. The pulmonary artery was clamped during viral infusion and the viral solution was not flushed out at the end of 60 minutes cold storage prior to performing heart transplantation. Viability of the grafts was verified daily by palpation of the beating transplanted heart.

Tissue Analysis

Transplanted hearts (n = 12 per group) were harvested 4 days after surgery. For PCR, RT-PCR, immunohistochemical staining and NOS activity assay, hearts (n = 6 per group except PCR for control, n=2.) were flushed with Phosphate-buffered saline solution and then cut into apical, mid ventricular

and basal sections. For assessment of ventricular function transplanted hearts (n = 6 per group) were removed and prepared for Langendorff perfusion.

Polymerase Chain Reaction (PCR)

The presence of recombinant eNOS DNA from the transduced hearts was analysed by PCR. Genomic DNA was prepared from apical and basal segments of eNOS (n=6) and LacZ transduced hearts, (n=2 as control) by standard proteinase K digestion in phenol and chloroform extraction conditions. Primer sequences utilised for the experiments were as follows: upper 5' AGG CGT CGG TGG GAG GTC TAT, lower 5' GCG CAC AGA GTG TCG TAG GTG ATG. The upper primer was complementary to the CMV promoter. PCR components include: 1 x PCR buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 200 uM of dNTP mix, 0.5M dimethyl sulfoxide (DMSO), 0.1 μM of each primer, and 2.5 units Taq DNA polymerase (Promega, Madison, WI). One microgram of DNA was amplified using 35 PCR cycles (94°C for 30 seconds,denaturation; 63°C for 30 seconds, annealing; and 72°C for 30 seconds, polymerisation). The PCR product amplified was 356 base pairs in length.

Reverse Transcriptase PCR (RT-PCR)

Expression of recombinant eNOS mRNA was detected by reverse transcriptase PCR. Total RNA was prepared from apical and basal segments

of transplanted hearts using RNA STAT-60 isolation reagent (Tel-Test "B", Friendswood, TX). Reverse transcription (RT) was performed using a SuperScript Preamplification System for First Strand cDNA Synthase Kit (Life Technologies Inc., Gaithersburg, MD). Total RNA was transcribed into cDNA using oligo(dT) and random hexamers priming methods according to the manufacturer's protocol. RT-generated cDNA encoding for the eNOS gene was amplified using 35 PCR cycles (94°C for 45 seconds, 60^0C for 45 seconds and 72^0C for 60 seconds). Sequences utilised for the experiments were as follow: upper 5' TCAACCAGTACTACAGCTCC, lower 5' GTGGTTGCAGATGTAGGTGA. These primers were derived from bovine eNOS sequence and they do not generate PCR product with endogenous rat eNOS. Primers designed to detect expression of Glyceraldehyde 3-Phosphate Dehydrogenase (G3PDH, Amplimer set, Clontech Laboratories, Inc., Palo Alto, CA) were used to test the efficiency of cDNA synthesis. The PCR product for eNOS migrated to 250 base pairs and the G3PDH at 450 base pairs.

Immunohistochemical staining

Midventricular cross sections of the transplanted hearts were embedded in OCT medium (Miles, Elkhart, Ind.) and snap frozen in a liquid nitrogen-cooled isopentane bath. Sections (5 μm thick) were cut at 25μm intervals, fixed for 10 minutes in cold acetone (4°C), fan-dried for 10 minutes

and further fixed in 1% paraformaldehyde/EDTA for 3 minutes. Endogenous peroxidase activity was blocked with 0.1 % sodium azide/0.3% H_20_2 for 10 minutes. Incubating sections with 5% goat serum/PBS-Tween 20 blocked non-specific protein binding sites. Then 5 μg/ml of anti-eNOS monoclonal antibody (N30020) (Transduction Laboratories, Lexington, KY) was added incubated for 60 minutes at room temperature. After rinsing, biotinylated rabbit anti-mouse F(ab')2 (1:300) was added for 20 minutes. After further incubation for 20 minutes with peroxidase conjugated-streptavidin (1:300), the slides were incubated for 30 seconds in 0.1 M sodium acetate buffer, pH 5.2. Then the slides were placed in AEC (3-amino-9-ethylcarbazole) substrate solution and incubated for 15 minutes at room temperature, counterstained in mercury-free haematoxylin for 1 minute and further rinsed for 3 minutes in cold running tap water before being mounted. Myocardial cells with immunoreactivity were then counted from all 5 sections of both control (LacZ) and experimental (eNOS) groups and a mean calculated for each heart.

Nitric Oxide Synthase Activity

NOS activities of LacZ (as control) and eNOS transduced hearts were determined by measuring the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline by methods originally described by Myatt[115] and modified by Barber[116].

Immediately after harvesting transplanted hearts, mid-ventricular sections were minced with surgical scissors and frozen at -70 °C until the time of assay (<30 days). For activity assay, the frozen tissue was diluted in 5 volumes of ice cold homogenisation buffer of the following composition: 50 mM Tris, 320 mM sucrose, 0.1 mM EDTA, 100 μg/ml phenylmethylsulfonyl, plus one tablet Complete™ (Boehringer Mannheim, Indianapolis, IN) protease inhibitor cocktail per 50 ml buffer, pH 7.8. Samples were homogenised on ice for 10 sec at full speed using a Tekmar tissue homogenizer (Tekmar, Cincinnati, OH). Homogenates were centrifuged at 2000 x g for 15 min at 4°C to remove cellular debris. The supernatant was passed onto an equilibrated 10-DG desalting column (Bio-Rad, Hercules, CA) and eluted according to the manufactures directions. A small aliquot was used to determine protein concentrations using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Protein homogenates were used immediately for determination of NOS activity.

To quantitate NOS activity, duplicate reactions were carried out in the presence of calcium (total activity), in the absence of calcium plus EGTA (calcium independent activity), and in the absence of calcium plus EGTA in the presence of N^G-monomethyl-l-arginine (L-NMMA) (non-specific activity). Reactions were started by adding 150 μl of protein homogenate to 150 μl of a cofactor mix such that final concentrations were as follows: 14.7 nM ^{3}H-l-arginine (0.3 μCi specific activity at 68 Ci/mmol), 5 μM l-arginine, 54 mM l-valine, 1.2 mM $MgCl_2$, 1 mM NADPH, 50 U/ml calmodulin, 2 μM

FAD, 10 μM tetrahydrobiopterin, and, as described above, with or without 0.83 mM $CaCl_2$, 1 mM EGTA, and 2 mM L-NMMA. The reaction was incubated on a shaker at 27°C for 1 hr and terminated by the addition of 1.5 mls ice cold stop buffer (20 mM HEPES, 8 mM EDTA, pH 5.5). Separation of ^{3}H-l-arginine from ^{3}H-l-citrulline was accomplished using affinity columns containing a resin which retains the charged species of ^{3}H-l-arginine while allowing ^{3}H-l-citrulline to pass through. Thus the assay mixture was passed over Poly-Prep chromatography columns (Bio-Rad) loaded with 1 ml of equilibrated AG 50W-X8 Na^+ form 200-400 mesh molecular biology grade resin (Bio-Rad) and the elute collected into 18 mls of Opti-Fluor (Packard, Meriden, CT). The column was washed with 2 mls of water while continuing to collect into the scintillation fluid. ^{3}H-l-citrulline activity was determined using a Beckman 6800 liquid scintillation counter. Activity calculations account for scintillation counting efficiency and the ratio of ^{3}H-l-arginine to non-radioactive l-arginine in the incubation mixture. Nitric oxide produced by NOS is presumably in a 1:1 molar ratio with l-citrulline and thus NOS activity is expressed as pmoles of ^{3}H-l-citrulline produced per mg of protein per hour. Calcium dependent activity equaled total activity minus calcium independent activity after correcting for non-specific activity.

Assessment of Left Ventricular Function

Functional effects of eNOS transduction on transplanted hearts were determined using the Langendorff preparation (Langendorff, 1895) (Figure 2.7). The set-up for Langendorff preparation was described in Chapter 2. Briefly, explanted eNOS and LacZ transduced hearts were arrested with cold saline, quickly excised and then mounted on the Langendorff apparatus and perfused immediately with Krebs buffer. Left ventricular function was expressed as left ventricular developed pressure (LVDP = left ventricular systolic pressure minus left ventricular diastolic pressure). Left ventricular function curves were constructed by progressively increasing the volume of the balloon to achieve end-diastolic pressures (LVEDP) of 0, 4, 8, 12, 16 and 20 mmHg. Following the assessment of cardiac function, the LVDP was maintained at the optimal level and the heart was allowed to stabilise for 5 minutes. Coronary flow was measured at 1, 2, 3, 4, 5, 10, 15, 20, and 25 minutes after beginning a continuous infusion of N^G-Monomethyl-L-Arginine, 10^{-4} M (L-NMMA). L-NMMA is an inhibitor of nitric oxide synthase. All data were collected and analysed.

Statistical Analysis

The quantitative data of number of cells stained positive for eNOS was expressed in median and range because of unequal variances. The data for NOS activity assay and functional studies suggested equal variances. Therefore,

results of NOS activity (picomoles/mg protein/hrs) were expressed as mean ± SEM; n represented the number of animals. NOS activity (picomoles/mg protein/hrs) of AdeNOS transduced hearts were compared against control (AdLacZ transduced) using a two-tailed Student's t-test for unpaired data. For analysis of left ventricular function, areas under the curves were compared by analysis of variance. Analysis of variance for repeated measurements was used to analyse changes in coronary flow. For post hoc analysis, Bonferroni correction was used when a significant F value was found. A P value less than 0.05 was considered significant.

Results

PCR

To confirm gene transfer, PCR was performed on DNA extracted from AdeNOS and AdLacZ transduced hearts. Bands at 356 base pairs corresponding to positive control for bovine eNOS were present in all lanes for AdeNOS transduced hearts (n=6). In contrast, no bands were seen in the controls (AdLacZ transduced hearts, n=2; Figure 6.1). The PCR product was subsequently sequenced by Dr O'Brien's group and determined to be bovine eNOS.

RT-PCR

To confirm transcription of the recombinant eNOS gene, transduced hearts were examined for the presence of eNOS mRNA by RT-PCR. Bands at 250 base-pairs corresponding to positive control for mRNA for eNOS were present in all lanes for eNOS transduced (n=6) but not in the AdLacZ transduced hearts (n=6, Figure 6.2). The reaction was negative in the absence of reverse transcriptase.

PCR and RT-PCR confirmed the presence of recombinant eNOS in the AdeNOS transduced hearts. As both these techniques were non-quantitative, the level of transgene expression was determined by NOS activity assay.

Immunohistochemical Staining

The presence of eNOS was detected by immunohistochemical staining. As the antibody to eNOS used was not specific for recombinant eNOS, endothelial cells in both the AdeNOS and the AdLacZ (control) groups were immunoreactive for eNOS. In the AdLacZ transduced hearts (control), the mean number of immunoreactive cardiomyocytes per section was 2 ± 1 (mean ± SE, Figure 6.3). In the AdeNOS transduced hearts, the mean number of immunoreactive cardiomyocytes per section was 254 ± 102 (mean ± SE, Figure 6.3). The majority of the eNOS positive staining was found in the endothelial cells of the capillaries in the AdLaZ group. The number of positive eNOS staining cardiomyocytes was significantly greater in the AdeNOS hearts

compared to AdLaZ hearts. This finding suggested that transgene expression may have contributed to the greater number of eNOS staining cardiomyocytes in the AdeNOS group.

NOS Activity

Total NOS activity was 41.7 ± 5.1 ρmoles L-[^{3}H]-citrulline/mg protein/hr in the AdLacZ transduced (control) group and 57.7 ± 5.2 ρmoles/mg protein/hr in the AdeNOS transduced group, (n=6 per group, P=0.05) (Figure 6.4). Calcium-dependent activity of NOS was 38.4 ± 4.7 ρmoles/mg protein/hr in the AdLacZ transduced group vs 53 ± 5 ρmoles/mg protein/hr in the eNOS transduced group (P = 0.05). Calcium-independent activity of NOS was 3.3 ± 0.5 ρmoles/mg protein/hr in the AdLacZ transduced group and 4.7 ± 1.5 ρmoles/mg protein/hr in the AdeNOS transduced group (P = NS).

Although the activity assay could not separate endogenous from recombinant eNOS, the results showed that calcium dependent activity of NOS was greater in the AdeNOS group compared to the AdLaZ group . This suggested that the increased in NOS activity in the AdeNOS was contributed by the overexpression of the eNOS transgene.

Cardiac functional assessment

Mean coronary flow after the initial period of stabilisation was 11.7 ± 0.5 ml/min in the AdLacZ group and 10.9 ± 0.7 ml/min in the AdeNOS group. There were no significant differences in cardiac function as measured by LVDP (left ventricular developed pressure) between the two groups (n=6 per group, Figure 6.5).

L-NMMA affected coronary flow comparably in both groups (Figure 6.6). During the initial period of L-NMMA infusion, coronary flow in all hearts increased transiently, but then decreased by 25 percent of baseline flow within two minutes.

Discussion

The aim of the present study was to demonstrate the feasibility and functional consequences of overexpressing eNOS in the transplanted heart using adenoviral-mediated gene transfer. Successful gene transfer and transgene expression in the transplanted heart was confirmed by PCR and RT-PCR. The significantly greater number of eNOS positive staining cardiomyocytes in the AdeNOS compared to the AdLaZ (control) group suggested the presence of recombinant eNOS in the former. This was further supported by the increased NOS activity in the AdeNOS group. The limitation of the immunostaining and activity assay was the inability to exclude the possibility

of induced endogenous eNOS expression. Recombinant gene expression however, did not affect ventricular function or coronary flow as assessed using a Langendorff preparation following heterotopic heart transplantation (non-loaded left ventricular chamber).

This study demonstrated the feasibility of using adenoviral vectors for gene transfer of a biologically relevant molecule to the transplanted heart. Adenoviral-mediated gene transfer was used because it has been shown to efficiently transduce non-replicative cells. Chapters 3 and 5 suggested that in the transplant setting, some of the limitations of this technology including the transient nature of transgene expression[117] and the difficulty of achieving prolonged luminal contact of vector in coronary vessels may be partially overcome. In this study as in Chapters 3 and 5, transgene expression was seen predominantly in cardiomyocytes. Interestingly, eNOS was also detected in small numbers of ventricular myocytes of AdLacZ transduced hearts. This observation is consistent with previous studies[118-120] showing the presence of eNOS in cardiomyocytes.

The potential effect of nitric oxide (NO) on myocardial function has been the subject of many studies with conflicting conclusions. The excessive and prolonged production of NO by inducible nitric oxide synthase (iNOS) in transplantation rejection and sepsis may be responsible for the negative inotropic effect on cardiac muscles[111,113,114]. In contrast, other studies on isolated heart preparations showed that increasing NO using NO donors resulted in enhanced cardiac contractility[121] and supplementation of NO during

the period of ischaemia-reperfusion has also been shown to have beneficial effects[122,123]. Furthermore, cardiac preservation in the heterotopic heart transplant model was enhanced by supplementation of the nitric oxide pathway[124]. In a recent study, the importance of basal NO production in preserving myocardial systolic function and coronary vascular tone was demonstrated[110]. Therefore, the role of eNOS in cardiomyocyte is unclear. To address this issue, we studied the effect of overexpression of eNOS on the contractility and coronary flow of the transplanted heart in a model in which eNOS overexpression was predominantly in cardiomyocytes. Using the Langendorff model, despite documented eNOS overexpression, this study did not show any significant difference in contractility and coronary flow between the eNOS and the LacZ transduced hearts. This negative result may suggest that eNOS is not involved in regulating cardiac contractility and coronary blood flow or that the distribution or efficiency of transgene expression was inadequate to affect these variables. If the latter possibility is true, more efficient means of transferring genes to the myocardium in the transplantation setting may affect cardiac contractility and coronary blood flow.

It has been proposed that eNOS gene transfer to the donor heart may be useful in the treatment or prevention of transplant-related atherosclerosis. This condition is the greatest limitation to long-term survival after heart transplantation and there is currently no effective treatment except re-transplantation. Endothelial nitric oxide is an important modulator of vascular smooth muscle tone and proliferation. Potential beneficial properties of nitric

oxide include inhibition of platelet aggregation, leukocyte adhesion and smooth muscle cells proliferation[106,107]. As smooth muscle cell proliferation forms an integral part of the pathologic process of accelerated transplant atherosclerosis,[6] overexpression of eNOS by adenoviral-mediated gene transfer leading to a controlled release of NO at the target area may modify this process[108,125] without compromising cardiac function and avoiding systemic effects. In this model, however, transgene expression was mainly detected in cardiomyocytes. In contrast, rare endothelial cells expressed the transgene. Therefore, it remains to be seen whether this pattern of overexpression of eNOS will influence intimal processes such as smooth muscle cell hyperplasia. However, NO is diffusible and the laboratory of Dr O'Brien has previously shown that adventitial expression of eNOS in the rabbit carotid artery or the canine basilar artery can effect vascular reactivity[48,126]. This suggests that eNOS overexpression outside the intima (e.g. in cardiomyocytes surrounding small vessels) may influence pathological processes involving the intima.

In summary, delivery of genes to the transplanted heart via the aortic root targets cardiomyocytes. This study demonstrated successful gene transfer of eNOS to the transplanted heart using PCR and RT-PCR. Immunohistochemistry and measurement of NOS activity suggested overexpression of eNOS Transgene in AdeNOS transduced hearts compared to controls. eNOS overexpression in cardiomyocytes in this model did not result in alteration of myocardial contractility or coronary blood flow. In the future, methods that enhance gene transfer efficiency may prove useful to modify

these variables in the donor organ. Whether or not transduction of cardiomyocytes will modify vascular consequences of transplantation remains to be determined.

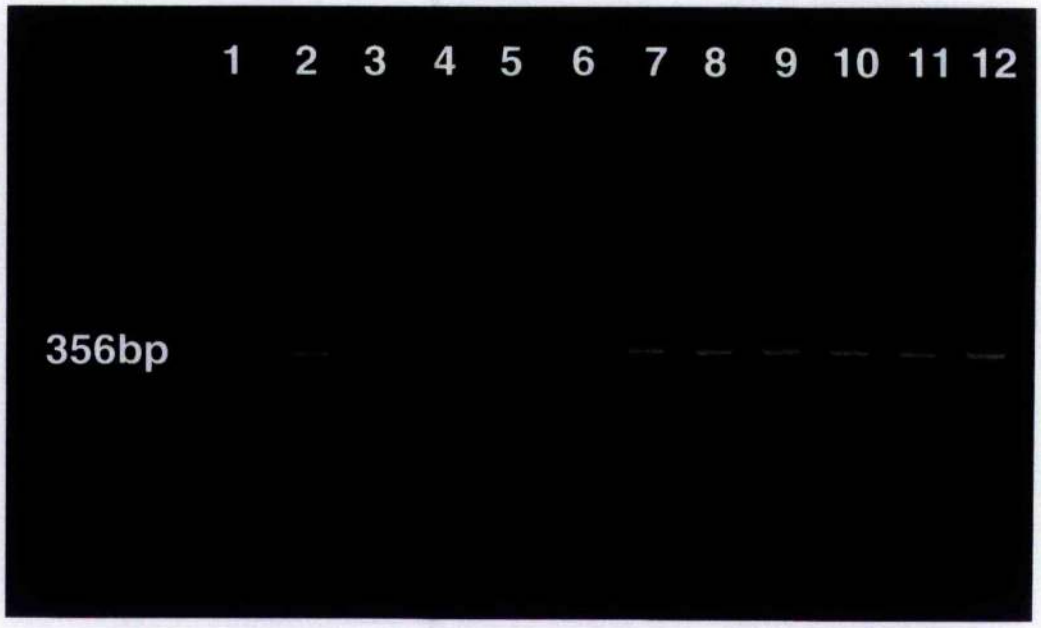

Figure 6.1: PCR to detect recombinant cDNA

PCR analysis to detect recombinant eNOS gene in AdeNOS and AdLacZ (control) transduced hearts using primers specific to the recombinant eNOS. Lane 1 is the reference ladder for number of base pairs. Lane 2 is the positive control (eNOS plasmid). Lanes 3 and 4 are negative controls. Lanes 5 and 6 represent AdLacZ transduced hearts (n=2, control). Lanes 7,8,9,10,11 and 12 represent AdeNOS transduced hearts(n=6). Positive bands confirming the presence of recombinant eNOS gene cDNA were observed only in Lanes 7 -12.

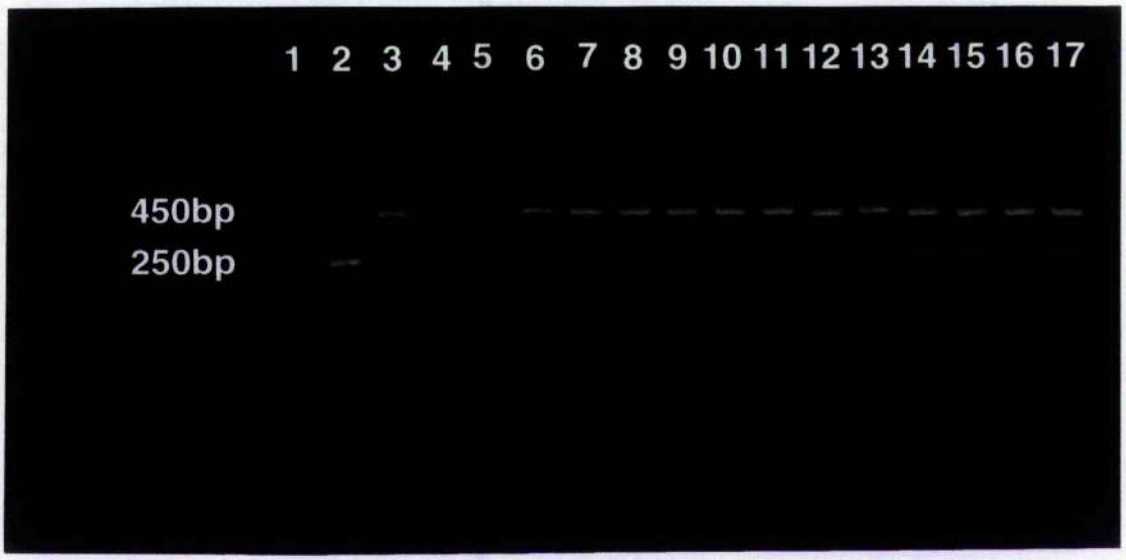

Figure 6.2: RT-PCR to detect recombinant mRNA

RT-PCR analysis to detect mRNA for recombinant eNOS in AdeNOS and AdLacZ (control) transduced hearts. Lane 1 represents the 100 base pairs DNA marker. Lane 2 represents positive signal at 250 base pairs in cDNA derived from plasmid with eNOS gene. Lane 3 represents positive signal at 450 base pairs for positive control (PCR product of Glyceraldehyde 3-Phosphate Dehydrogenase). Lane 4 represents negative signal at 661 base pairs in cDNA derived from plasmid with inducible NOS (iNOS). Lane 5 = water. Lane 6 to 11 = controls (n=6, LacZ transduced hearts) showing no signal for eNOS mRNA. Lane 12 to 17 = eNOS transduced hearts (n=6) showing positive signals for eNOS mRNA. These primers were derived from bovine eNOS sequence and they do not generate PCR product with endogenous eNOS.

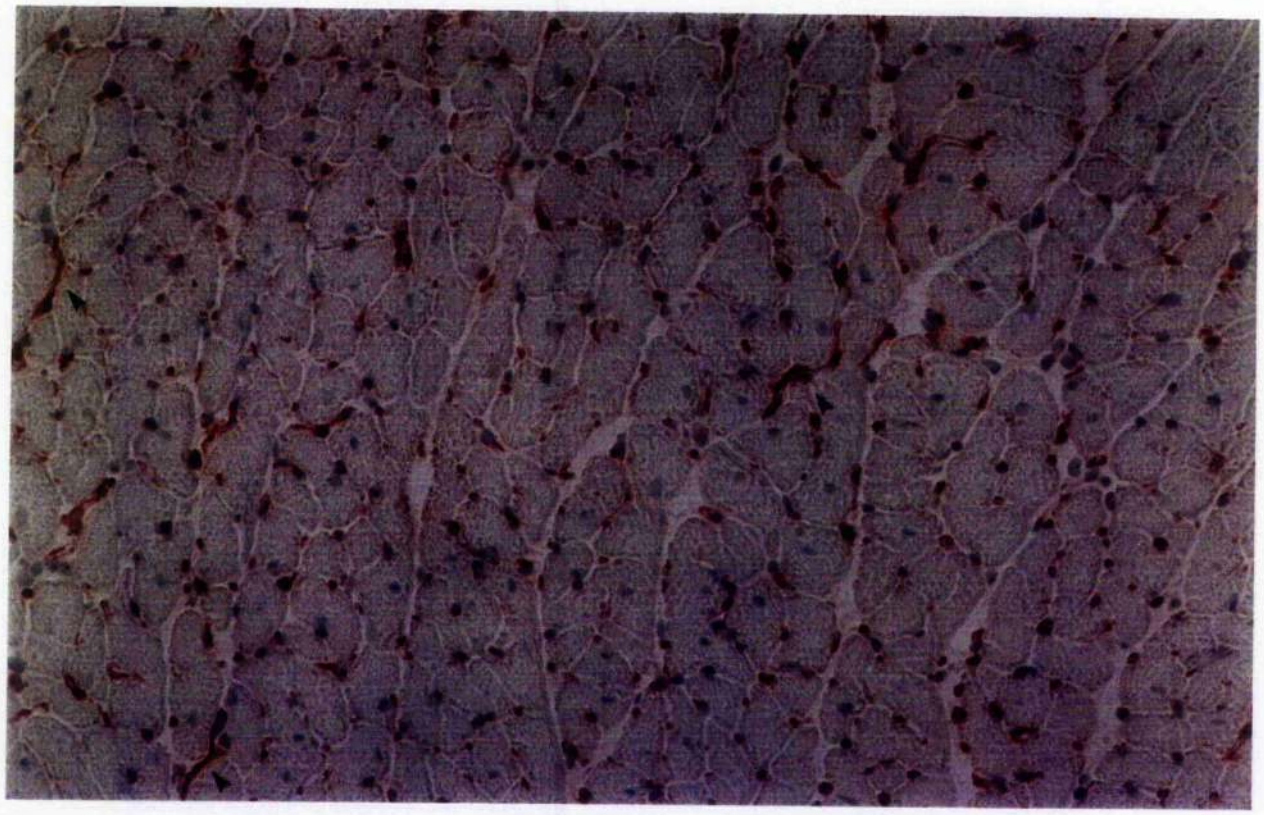

Figure 6.3: Immunohistochemical staining of AdLacZ transduced hearts

Immunohistochemical staining of 5μm section of the midventricular section of AdLacZ transduced heart using monoclonal antibody to eNOS. Arrows mark positive staining (brown) in the endothelial surfaces of vessels including capillaries.

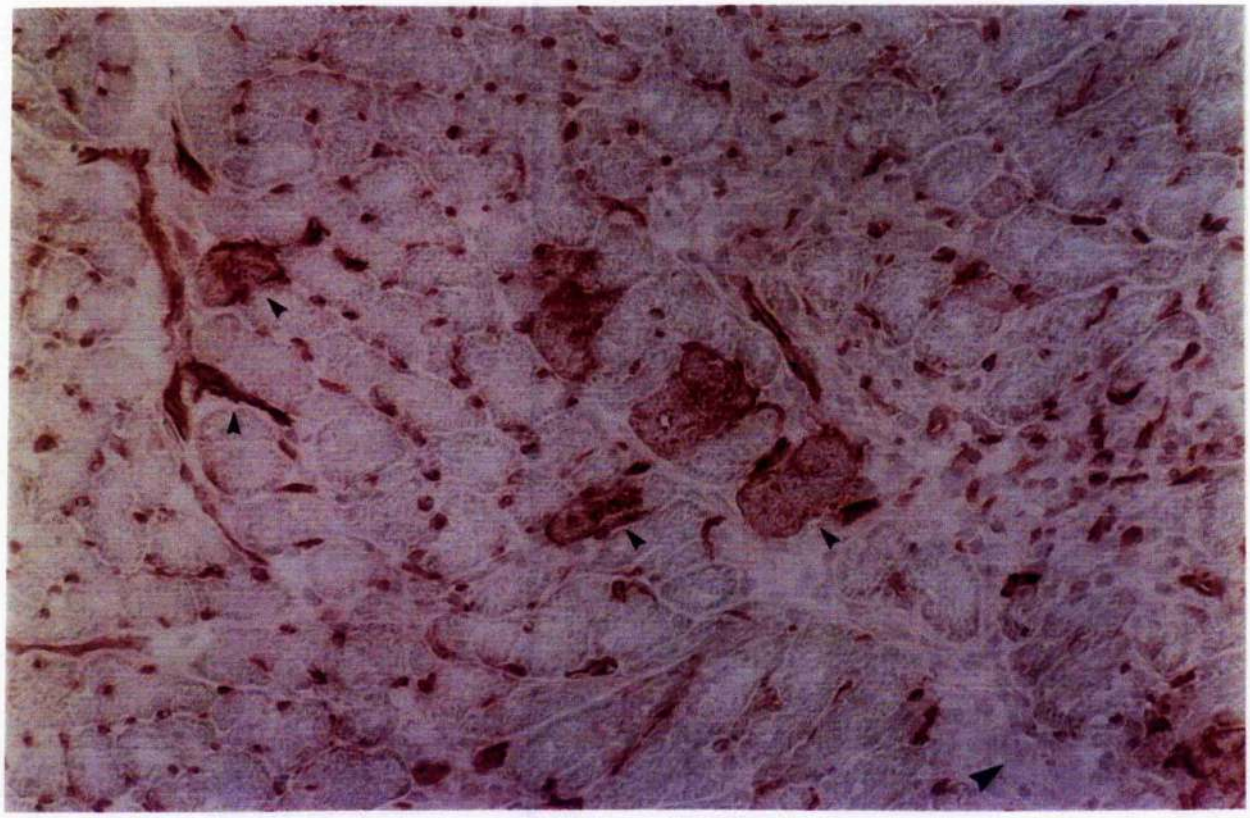

Figure 6.4: Immunohistochemical staining of AdeNOS transduced hearts

Immunohistochemical staining of 5μm section of the midventricular section of AdeNOS transduced heart using monoclonal antibody to eNOS. Small arrows mark positive staining (brown) in the endothelial surfaces of vessels including capillaries and cardiomyocytes. There is an area of organising ischaemia in the lower right-hand corner (large arrow).

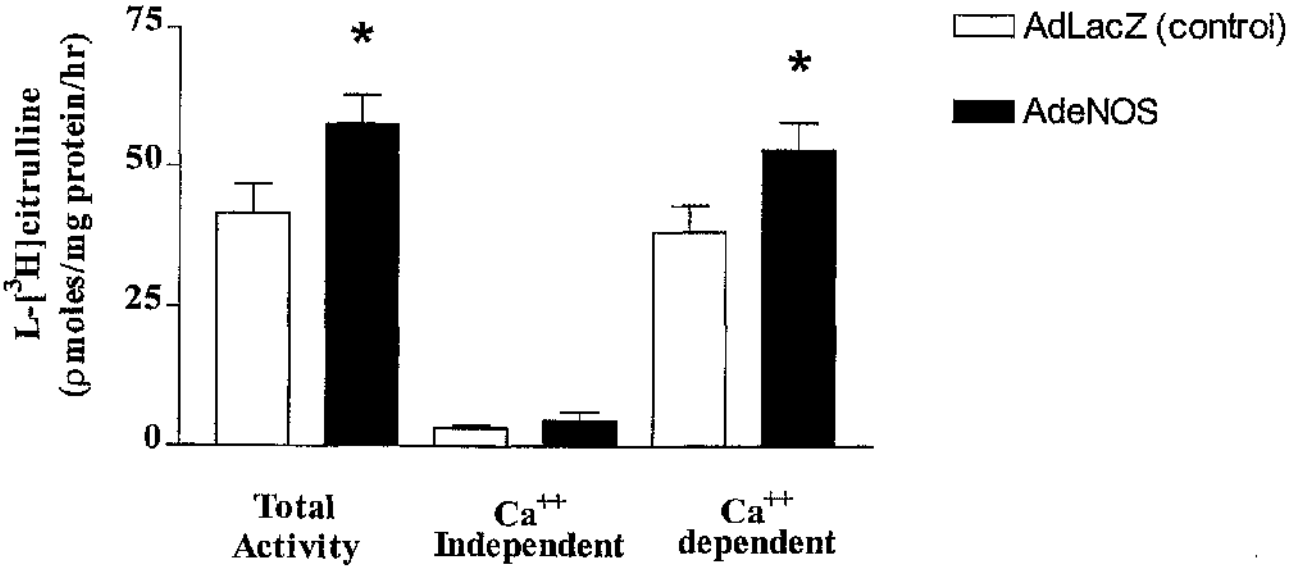

Figure 6.5: NOS activity assay

NOS activity of AdLacZ and AdeNOS transduced hearts determined by measuring the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline, expressed as mean ± SEM in pmoles/mg protein/hr (n=6 in each group). The AdeNOS transduced hearts showed greater level of total and calcium-dependent NOS activity compared to AdLacZ transduced hearts, (two-tailed Student's t- test, P = 0.05). Calcium-independent NOS activity was similar in both groups.

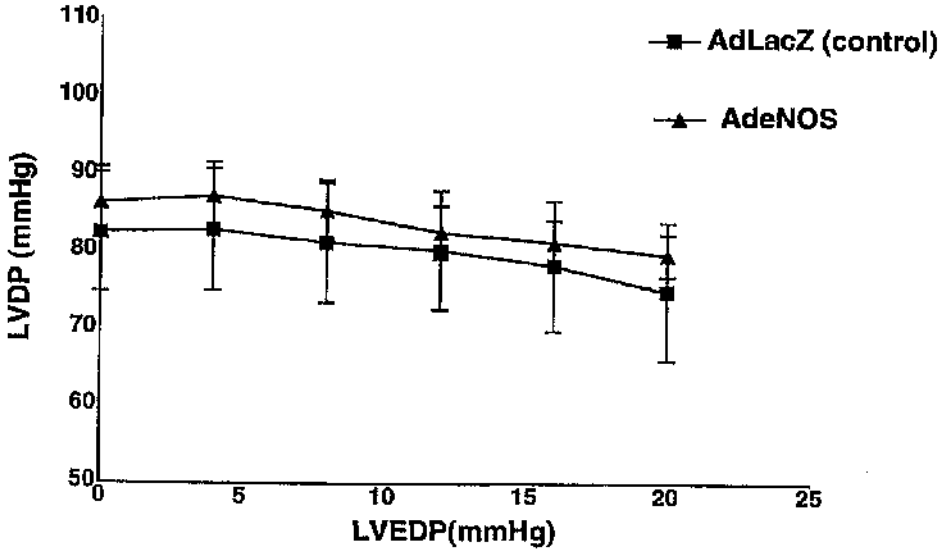

Figure 6.6: Assessment of left ventricular function in transduced hearts

Four days after gene transfer and transplantation, AdeNOS and AdLacZ transduced hearts were explanted and left ventricular function assessed by Langendorff preparation. Left ventricular function was expressed as LVDP mmHg (left ventricular developed pressure, mean ± SEM. No significant difference in LVDP was detected between AdLacZ and AdeNOS transduced hearts (n=6 per group).

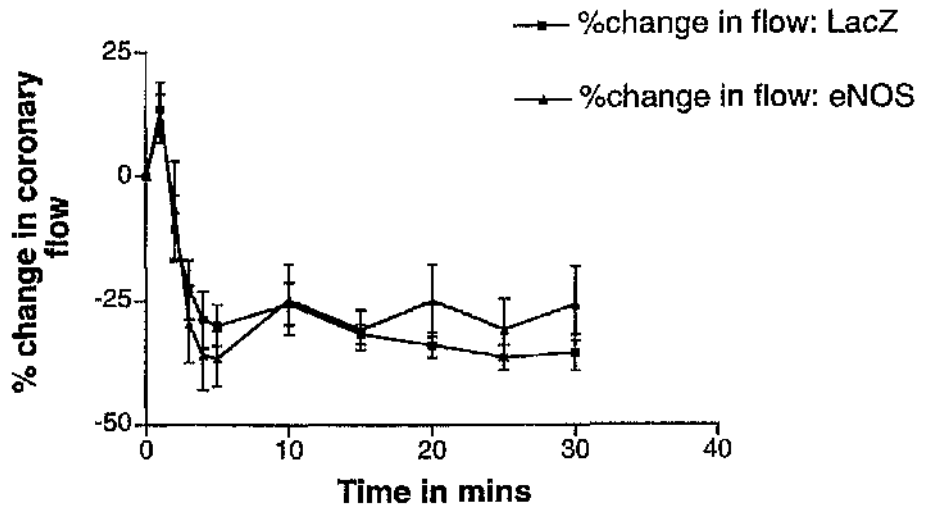

Figure 6.7: Assessment of coronary flow in transduced hearts

Four days after gene transfer and transplantation, AdeNOS and AdLacZ transduced hearts were explanted and coronary flow was assessed using an in-flow meter in the Langendorff preparation. Changes in coronary arterial flow following L-NMMA infusion (10^{-4} mmol) were recorded. Results were expressed as mean ± SEM % change in flow (ml/min, n=6 per group) from baseline flow rate (before L-NMMA infusion). Infusion of L-NMMA caused comparable changes in coronary blood flow in AdLacZ and AdeNOS transduced hearts.

Chapter 7 : Conclusions

Summary and Future Directions

This thesis addressed many of the basic issues in adenoviral-mediated gene transfer to the transplanted heart. The potential for gene transfer in the heart transplant setting is exciting. If this technique is successful, then it may prove to be an extremely valuable tool in the study of the biology of heart transplantation. With better understanding of the pathogenesis of the disease processes, strategies for therapeutics could be realised.

In Chapter 1, a brief introduction to heart transplantation and the challenges faced were discussed. The various vector systems for gene transfer were described and the advantages and disadvantages of these vectors discussed. The most efficient vectors to date for gene transfer are the adenoviral vectors. They can transduce non-replicative cells and are thus ideally suited as vectors for the heart.

Chapter 2 defined the aims of the study. The animal model used and the technical training were described. The biosafety concerns of adenoviral vectors with emphasis on precautions for working with these agents were also discussed. This would be a significant issue in clinical trials.

Chapter 3 and 4 studied the feasibility of transferring genes to the transplanted hearts and examined the conditions that may favour efficient gene transfer. When this study was initiated, very little was known of gene transfer in the transplanted heart. The results suggested the importance of coronary

distension during viral administration on the efficiency of gene transfer. The role of distending pressure in facilitating cardiovascular gene transfer has also been shown in other studies of gene transfer to the vasculature[58,127]. A method of vector delivery which resulted in relative efficiency of gene transfer was established in this chapter which facilitated subsequent studies. Also it was noted that there were considerable inter-animal variability in the results. This may be due to the biological variability of gene transfer or a reflection of the method of vector delivery. If it is the latter, then alternative techniques of vector delivery, for example recirculating infusion of the vector, should be explored.

A major limitation in adenoviral-mediated gene transfer is the transient nature of transgene expression. In addition, the effectiveness of a second administration is curtailed with the formation of neutralising antibodies. This may limit the usefulness of adenoviral vectors in treating chronic diseases. Chapter 5 investigated the effects of immunosuppression, which is routinely used in clinical transplantation to prevent rejection, on duration of gene transfer in both syngeneic and allogeneic transplant models. It showed a moderate prolongation of transgene expression. Whether this moderate prolongation of duration of transgene expression is adequate for modifying disease processes affecting transplanted hearts warrants further investigation.

In Chapter 6, the feasibility of transferring an important biological gene to the transplanted heart was confirmed. The limitation of the study in Chapter 6 was the lack of distinction between endogenous eNOS expression

which may be upregulated and the overexpression of eNOS by the recombinant eNOS transgene. However, the data presented was supportive of the eNOS transgene being overexpressed in the studied hearts. The effects of overexpressing nitric oxide synthase on these hearts were studied. Nitric oxide has a number of beneficial properties, including inhibition of smooth muscle cell proliferation, that may modify the pathogenesis of accelerated transplant atherosclerosis. This warrants further investigation in an experimental model of heart transplant atherosclerosis.

In conclusion, gene transfer to the transplanted heart is feasible. The success of gene transfer is dependent on the efficiency of the delivery vectors. An ideal vector would efficiently deliver genes to the appropriate cell type, result in long-lived regulated transgene expression and avoid toxicity. The current vectors fall far short of these goals. Further development and advances in vector design may help fulfil these requirements. Therefore, with the ideal vector transferring a therapeutic gene to an appropriate target cell type or organ, gene therapy may favourably modulate immune responses and enhances graft function in clinical heart transplantation.

The Current Status Of Human Gene Therapy:

Human gene therapy has been heralded for its potential to revolutionise modern medicine through the use of recombinant DNA technology to treat

inherited and acquired diseases. The first human gene therapy clinical trial[25] was performed on two girls with inherited adenosine deaminase deficiency (ADA) in 1990. Although the results of this trial were difficult to interpret, due a lack of controls and the patients continued receiving standard therapy, the observations were consistent with the conclusion that this strategy of ex vivo gene transfer evoked biological responses that were relevant to the treatment of ADA deficiency. Since then, the number of clinical protocols world wide have increased dramatically. By 1996, there were at least 232 protocols[128] approved for clinical trials. The majority of the trials involves inherited diseases (including cystic fibrosis), cancer and infectious diseases. Recently, a clinical gene therapy trial of the heart was initiated[129]. A naked plasmid DNA encoding for the vascular endothelial growth factor (VEGF) gene was directly injected into ischaemic areas of the myocardium to stimulate angiogenesis. Early results were encouraging.

However, the initial enthusiasm surrounding the potential role of gene therapy in the treatment of human disease has now been tempered by the realism of the current situation. Significant obstacles to success include the inconsistency of results, vector production and the lack of an ideal vector[130].

The potential applications of human gene transfer are broad due the wide variety of diseases. The ideal vector will likely to be different for each application. Clinical experience to date suggests that adenovirus, retrovirus and plasmid-liposome vectors all require further refinement. Recently, new

generation of adenoviral vectors are becoming available. These vectors have less viral sequence and are therefore less toxic and results in more prolonged transgene expression. Therefore, the future for human gene therapy remains promising.

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Appendix

Publications

Immunosuppression Prolongs Adenoviral-Mediated Transgene Expression in Cardiac Allograft Transplantation

J Yap, T O'Brien, H D Tazelaar, C G A McGregor.

Cardiovascular Research 35 (1997) 529 - 535.

Distribution and Function of Recombinant Endothelial Nitric Oxide Synthase in Transplanted Hearts

J. Yap, T. O'Brien, D.A. Barber, H.D. Trazelaar, S.R. Severson, V.M. Miller, C.G.A. McGregor

Cardiovascular Research, *in press*

Influence of Temperature on Adenovirus-mediated Gene Transfer

Pellegrini C, O'Brien T, Jeppsson A, Fitzpatrick LA, **Yap J**, Tazelaar HD, McGregor CGA.

European Journal of Cardio-thoracic Surgery 1998; 13:599-603.

Conditions of Vector Delivery Improve Efficiency of Adenoviral-mediated Gene Transfer to the Transplanted Heart

J Yap, C. Pellegrini, T O'Brien, H D Tazelaar, C G A McGregor.

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Presentations and Abstracts

69th Scientific Conference of the American Heart Association, November 1996

Optimization for Gene Transfer in the Transplanted heart.

J Yap, T O'Brien, H D Tazelaar, C G A McGregor.

Circulation (Suppl) 1996; 94: 8: I-53

International Heart and Lung Transplantation Conference, London, April 1997

Immunosuppression Prolongs Adenoviral-mediated Transgene Expression in Cardiac Allograft Transplantation.

J. Yap, T O'Brien, H D Tazelaar, C G A McGregor.

Journal of Heart and Lung Transplantation 1997, 16;1: 63.

Society of Cardiothoracic Surgeons of GB & Ireland, 1997 Annual Meeting

Adenoviral-Mediated *Ex Vivo* Gene Transfer to the Transplanted Heart:

J. Yap, T O'Brien, H D Tazelaar, C G A McGregor.

Cardiac Surgery 97, National Heart and Lung Institute, Imperial College & Royal Brompton Hospital

Genetic Manipulation of the Transplanted Heart in a Rat Model

J. Yap