Adenovirus Mediated Gene Transfer of Skeletal Troponin C to Myocardium

A thesis submitted in fulfilment

of the degree of

Doctor of Philosophy

By

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Abbreviations

AAV	Adeno Associated Virus
Akt	protein kinase B
CHD	Coronary Heart Disease
CMV	Cytomegalovirus
cTnC	Cardiac Troponin C
DAB	3',3'-diaminobenzadine
DMSO	Dimethyl Sulphoxide
DOTAP	liposome formulation of a monocationic lipid (N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium propane methylsulfate in sterile water
GFP	Green Fluorescent Protein
HA	haemagglutinin antigen
HEK293	Human Embryonic Kidney 293 Cell line
IGF-1	Insulin-like growth factor-1
IHD	Ischaemic Heart Disease
Kb	Kilo base
pfu	plaque forming units
PBS	Phosphate Buffered Saline
sTnC	Skeletal Troponin C
SERCA	Sarcoplasmic Reticulum Calcium ATPase
TM	Tropomyosin
TnI	Troponin I
TnT	Troponin T

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Declaration

Assistance was provided by Niall Whyte who cut and mounted all histological sections. Jamie Varghese snared the isolated trabeculae and assisted with the calcium sensitivity experiments. Otherwise, all of the experimental work contained within this thesis was undertaken by me. The material has not been submitted previously for any other degree. Some of the results presented have been published during the period of study.

Publications

A Docherty, MN Hicks, GL Smith, SM Cobbe, HM Prentice. Delivering adenoviral vectors to rabbit myocardium: effect of dose and method of delivery on efficiency and micro-infarction. European Heart Journal 1999; 20 (Abstr. Suppl.): 249.

Summary

Ischaemic heart disease and the syndrome of congestive cardiac failure are increasing in prevalence within western societies. The limitations of present day interventions are discussed. There is a need to identify new therapeutic targets and the potential of molecular genetic methods is discussed. Experimental data from transgenic mice expressing skeletal troponin C is considered together with data from substitution of troponin isoforms in isolated preparation. The function of cardiac troponin C is significantly affected by intracellular acidosis and this is largely responsible for the early decoupling of the calcium transient and contractile dysfunction. In comparison skeletal troponin C is relatively unaffected by moderate degrees of intracellular acidosis. This led to the hypothesis that by displacing a proportion of the cardiac troponin C with the skeletal isoform, there would be the possibility of rendering cardiac muscle relatively resistant to the effects of intracellular acidosis. Perhaps such altered myocardium would be able to contract for longer in conditions of ischaemia.

Vectors for gene transfer are considered and the attractive properties of adenoviral vectors detailed. Routes by which to deliver vector to the heart are discussed and particular consideration is given to the intra-coronary injection of adenoviral vector.

Chapter 3 deals with the production and quality assurance of replication defective adenoviral vectors. The "supernatant rescue assay" method to detect wild type contamination is discussed but not utilised. Further experiments depend on the adenoviral vector which is free of wild type contamination. Although virus was propagated from single plaques and the

viral DNA tested by restriction enzyme analysis, the possibility of wild type contamination remained. This was to undermine the value of the data from subsequent experiments. Chapter 4 details the failure of the Swan-Ganz method of viral delivery, which in part may have been due to contamination of vector by replication competent wild-type virus.

A selective coronary injection method of vector delivery was achieved and then compared with an aortic cross-clamp method of vector delivery. The experimental design permitted a direct comparison of the efficiency of both methods. The selective coronary injection method was found to be more efficient. Neither method achieved adequate efficiency of gene transfer to allow investigation of genetically altered contractile physiology by means of a working heart preparation. The selective coronary injection method achieved foreign gene expression in up to 30% of myocytes in the trabeculae rich basal right ventricle. Pre-injection of serotonin before injection of low dose virus resulted in more myocardial damage than expected. The lack of a control group, injected with only vehicle, limited the value of these data. Chapter 7 details the results of physiological investigation of right ventricular trabeculae, isolated from rabbits following selective coronary injection of adenoviral vector encoding either beta-galactosidase or skeletal troponin C. The results are negative and the possible reasons discussed. The vector used for these experiments encoded a skeletal troponin C mutant where the final 9 amino-acids at the C-terminal had been deleted and replaced with a 12 amino-acid sequence which included a haemagglutinin antigen motif. In retrospect, confirmation of the integration of the gene product into the sarcomere should have been undertaken prior to proceeding to *in vivo* investigation. The final chapter places the results of this work in the context of a rapidly advancing field.

Chapter 1
General Introduction

Epidemiology of Ischaemic Heart Disease and Heart Failure.

Introduction

Ischaemic heart disease results from the atheromatous degeneration and the consequent narrowing of coronary arteries (Ross 1999). When the diameter of a coronary artery is reduced by more than 60%, this has an adverse effect on the coronary flow reserve and can result in an imbalance between myocardial oxygen supply and oxygen demand resulting in myocardial ischaemia. (Klocke 1987). This presents as the clinical syndrome of angina pectoris. Affected individuals can be limited in their physical activities by anginal chest pain or exertional breathlessness. Patients with coronary artery disease are at risk of myocardial infarction and subsequent congestive cardiac failure. The term "heart failure" is commonly applied to patients with impaired left ventricular systolic function. Although ischaemic heart disease is a common cause of heart failure, other causes include hypertension, valvular heart disease, infective, metabolic and endocrine disorders (Andersson & Waagstein 1993). Patients with the clinical syndrome of heart failure can present with symptoms of impaired exercise capacity, exertional dyspnoea, orthopnoea, paroxysmal nocturnal dyspnoea and peripheral oedema. Survival after onset of clinically apparent chronic heart failure is poor (Ho et al. 1993). In men, the median survival is only 1.7 years with a 5-year survival of only 25%. For women, the outlook is moderately better with a median survival of 3.2 years and a 5-year

survival of 38%. Although the commonest cause of symptomatic heart failure is systolic dysfunction, a similar clinical presentation can be found in left ventricular diastolic dysfunction and pericardial disease (Vasan et al. 1999). Symptomatic diastolic dysfunction is associated with an annual mortality of 19% for males and 9% for females. There are ongoing clinical trials in patients who are thought to have diastolic dysfunction but as yet there is no intervention which is proven to improve survival (Cleland 1999; Swedberg et al. 1999).

There are well-recognised modifiable risk factors and risk predictors for both ischaemic heart disease and heart failure. Predictors for the development of ischaemic heart disease included male gender, smoking, obesity, family history, age over 45 years, diabetes mellitus, hypertension and hyperlipidaemia.

Prevalence of Ischaemic Heart Disease.

Ischaemic heart disease and congestive cardiac failure are common in the western world. Although the death rate for ischaemic heart disease is in decline, it still accounts for more than one third of all deaths in adults over 35 years of age (Feinleib 1995). Table 1.1 shows the prevalence of self-reported myocardial infarction and symptomatic ischaemic heart disease as derived from the National Health and Nutrition Surveys (NHANES, American Heart Association. Heart and Stroke Facts: 1995 Statistical Supplement. American Heart Association 1994; Dallas TX 75231)

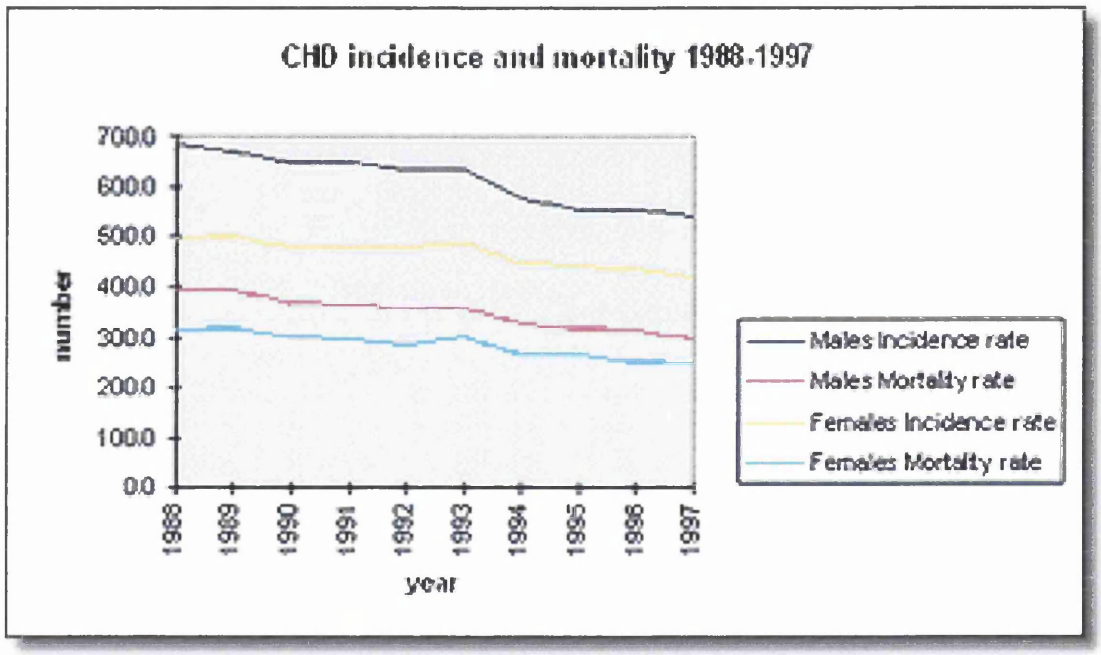
Table 1.1. Prevalence of self-reported myocardial infarction and symptomatic ischaemic heart disease.

Age Group (yrs)	Male (%)	Female (%)
40-49	7	5
50-59	13	8
60-69	16	11
70-79	22	14

These data are from a North American population but the increasing prevalence of disease with age is similar to that found in British cohorts. In the United Kingdom as a whole, death rates from ischaemic heart disease over a recent ten-year period have fallen by over 40% (BHF, Coronary Heart Disease Statistics. BHF statistics database 1998). In Scotland, the CHD death rate fell by approximately 30% in the years 1975 to 1994 (Capewell, Morrison, & McMurray 1999). Half of this improvement was attributable to measurable risk factor reduction (predominantly smoking cessation) and forty percent was attributable to medical treatments. The cumulative risk of developing ischaemic heart disease and congestive cardiac failure increase with age (Lloyd-Jones et al. 1999). For healthy men aged 40 years, the lifetime risk of developing coronary artery disease is one in two. Women lag behind men by about 10 yrs and for a 40-year-old woman the lifetime risk is one in three.

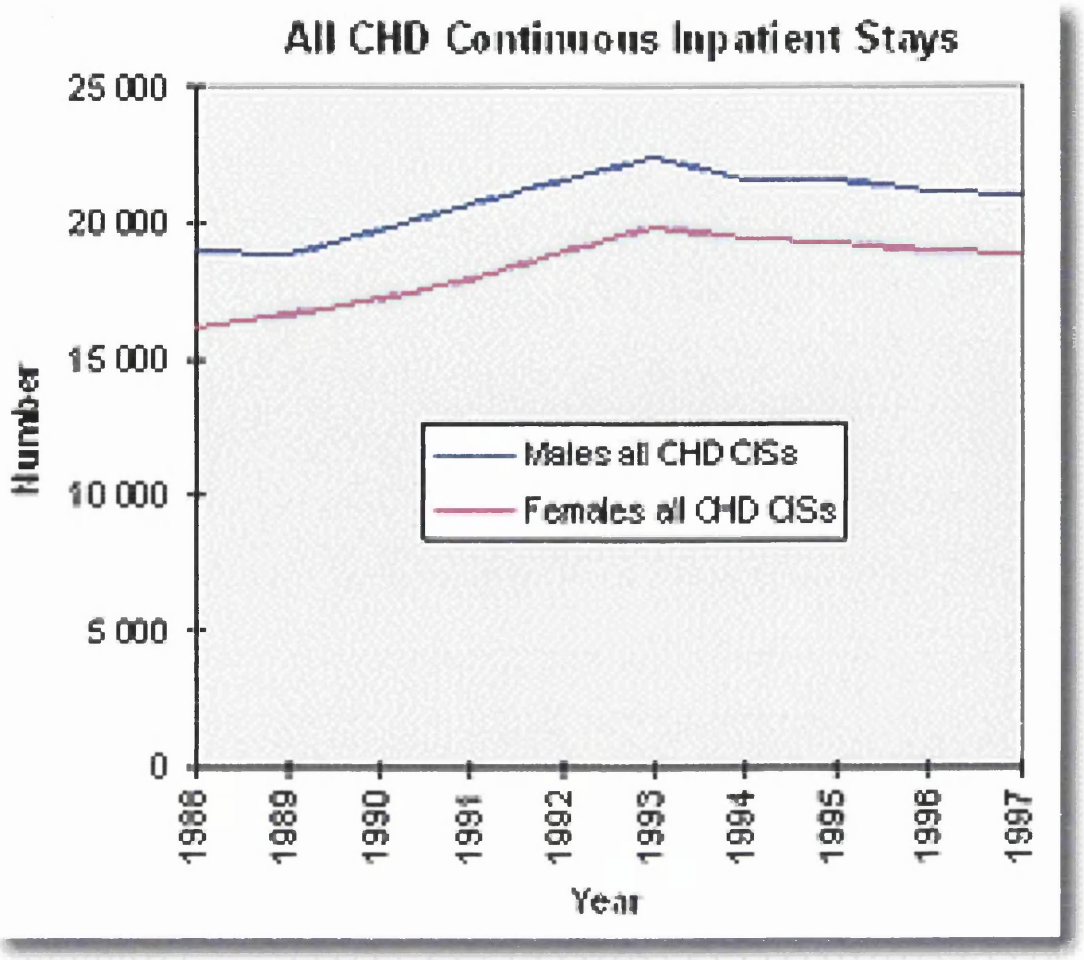
The Incidence of Coronary Heart Disease is in Decline.

The Scottish incidence of coronary heart disease has been declining and this has been paralleled by a reduction in mortality (Graph 1.1. Information and Statistics Division Common Services Agency for the NHS in Scotland).



Graph 1.1. Coronary Heart Disease Incidence and Mortality 1988-1997

Although there has been a decline in mortality, this is offset by the increasing age of the population. The net effect of these factors is a likely increase in the prevalence of CHD. In recent years the number of patients admitted to Scottish hospitals for treatment of CHD has increased (Graph 1.2, Information and Statistics Division Common Services Agency for the NHS in Scotland).



Graph 1.2. All Coronary Heart Disease Continuous Inpatient Stays

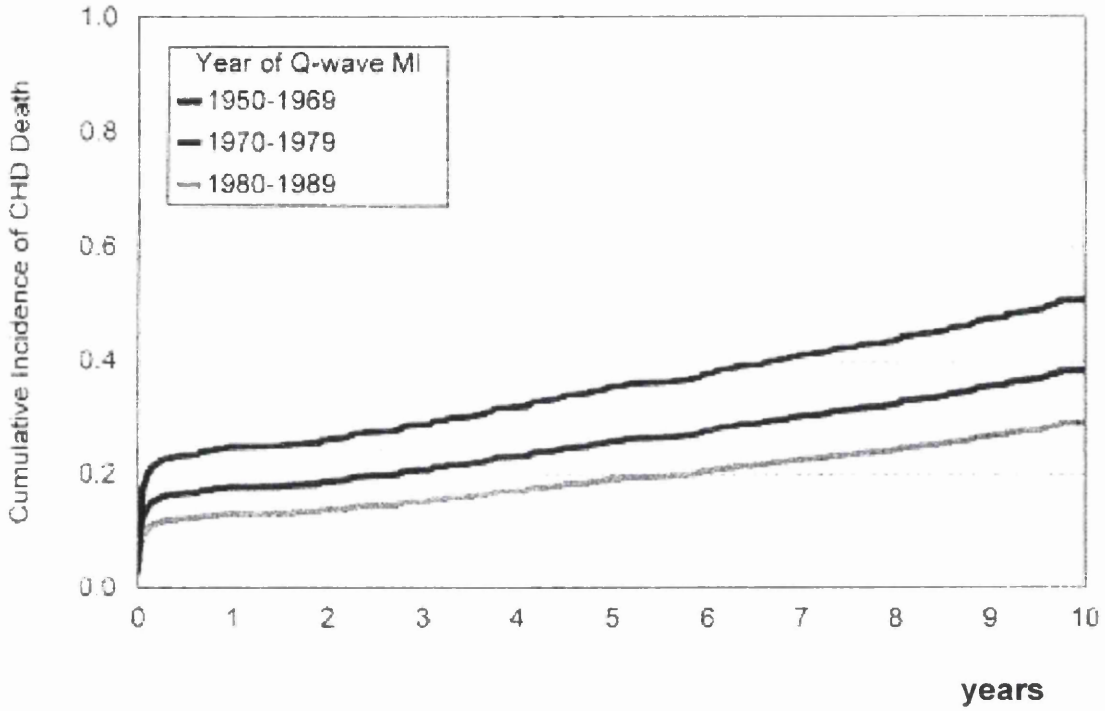
The same is true for the North American population, where the decline in incidence of CHD is offset by the age-adjusted decline in death rates from heart disease (Feinleib 1995). Consequently, the prevalence of ischaemic heart disease and congestive cardiac failure is likely to increase, with an associated increase in demand on healthcare resources (McMurray et al. 1998).

In the healthy population, the incidence of heart disease can be reduced by modification of risk factors. Smoking cessation has been associated with a 36% reduction in heart disease (Capewell, Morrison, & Mc Murray 1999). In patients with hypertension, treatment can reduce the incidence of stroke and

coronary heart disease by more than 30% (Curb et al. 1996). In hyperlipidaemia, asymptomatic healthy men at risk of developing coronary artery disease showed a significant reduction in end points when treated with lipid lowering therapy (Downs et al. 1998; Shepherd et al. 1995). Glycaemic control in diabetics has been shown to reduce microvascular end points (UK Prospective Diabetes Study Group 1998). In addition, diet and exercise are thought to be important (Krauss et al. 1998; Lemaitre et al. 1999).

In patients with established cardiovascular disease, reductions in mortality and morbidity have been shown in patients where modifiable risk factors have been addressed. For the same relative risk reduction as applied to primary prevention groups, fewer patients need to be treated to achieve one life saved. There is evidence for the benefit of smoking cessation (Wilhelmsson et al. 1975), treatment of hypertension (Connolly et al. 1983), treatment of hyperlipidaemia (Scandinavian Simvastatin Survival Study 1994), glycaemic control (Malmberg 1997), exercise (Leon et al. 1987) and diet (Burr et al 1989) in patients with established cardiovascular disease. Medical treatments demonstrated to reduce the attrition rate include Aspirin (Krumholz et al. 1996; Antithrombotic Trialists' Collaboration 2002), Beta-blockers and ACE inhibitors (CIBIS-II Investigators 1999, Pfeffer et al. 1992). In part due to these interventions, there has been a reduction in the cumulative incidence of coronary heart disease death after an initial Q-wave myocardial infarction (Graph 1.3, Guidry et al. 1999).

Graph 1.3 Cumulative Incidence of Coronary Heart Disease Death 1950-1989 (adapted from Guidry et al. 1999)



Nevertheless, in the United Kingdom, there is room for improvement in the secondary prevention of cardiovascular disease. In the UK ASPIRE study, up to a quarter of patients remained hypertensive, males more severely so than females. Over three quarters had a total cholesterol greater than 5.2 mmol/l. In patients on medication for blood pressure, cholesterol or glucose, risk factor profiles were little better than in those who were not. Only about one patient in three was taking a beta-blocker after myocardial infarction. Up to a fifth of patients who had had acute myocardial ischaemia were not taking aspirin at follow up (Bowker et al. 1996).

Therapeutic Options for Established Ischaemic Heart Disease.

Medical therapy, percutaneous coronary intervention and coronary artery bypass surgery are the three treatments found to effectively relieve the symptoms of ischaemic heart disease. In patients with stable angina the one-year incidence of death, myocardial infarction or admission to hospital with unstable angina is approximately 5% (Murphy et al. 1977). For patients with newly diagnosed unstable angina the combined end point of death, myocardial infarction or re-admission at six months is around 12% (Yusuf et al. 1998). In selected patients, percutaneous coronary intervention can reduce early and medium term unplanned admission to hospital with acute coronary syndromes, but has not been shown to reduce long-term mortality (FRISC II Investigators 1999). Selected subgroups may obtain a mortality benefit from coronary artery bypass surgery. There is data supporting a mortality benefit in patients with significant left-main coronary artery stenosis and in patients with three-vessel coronary artery disease and moderately impaired left ventricular function (Alderman et al. 1983). However, much of this data is from the CASS registry and is not randomised data. At 15 year follow-up there is no discernible mortality advantage in patients who had preserved left ventricular systolic function (Yusuf et al. 1994; Caracciolo et al. 1995a; Caracciolo et al. 1995b).

Therapeutic Options for Established Heart Failure

Several studies have shown that oral inotropic agents with vasodilatory properties such as amrinone, milrinone, enoximone, and xamoterol are associated with increased mortality rates irrespective of the cause of heart failure and its severity (Xamoterol in Severe Heart Failure Study Group 1990; Massie et al. 1985; Packer et al. 1991; Uretsky et al. 1990).The same is true for vesnarinone and ibopamine (Hampton et al. 1997; Cohn JN. VEST results. Presented at the Twelfth Plenary Session of the Sixty-ninth Scientific Session of the American Heart Association, New Orleans, LA: Nov. 10-13, 1996)

In the syndrome of heart failure secondary to left ventricular systolic dysfunction there is pathological neuro-endocrine activation. Angiotensin converting enzyme inhibitors, Beta-blockers and the aldosterone antagonist spironolactone have all been shown to reduce mortality in such patients. In addition there is evidence to show that these agents improve patient well-being and functional capacity in the long term. The reduction in mortality for ACE inhibitors and Beta-blockers is additive (SOLVD Investigators 1992; CIBIS-II Investigators 1999).

On-going Clinical Research

In heart failure, there is on-going clinical research into the use of neutral endopeptidase inhibitors (Rouleau et al. 2000) OVERTURE (Omapatrilat Versus Enalapril Randomized Trial of Utility in Reducing Events), endothelin antagonists and aldosterone antagonists (the EPHESUS, or Eplerenone Neurohormonal Efficacy and Survival Study), all of which may further modulate neuro-endocrine responses to heart failure. TNF-alpha modulators are under investigation (Aukrust et al. 1999). Calcium sensitising drugs are being investigated for their potential to increase cardiac force of contraction without increasing oxygen demand or causing arrhythmias. Two large studies of ACE inhibition and/or angiotensin II antagonism are ongoing which include patients with the clinical syndrome of heart failure, normal systolic function, and possible diastolic dysfunction (Cleland1999; Swedberg et al. 1999).

New Molecular Targets for Intervention

Introduction

Despite present therapeutic options, heart failure leads to a poor quality of life and has a five-year survival rate worse than many cancers. There is a need to uncover therapeutic targets out with the neuro-endocrine system.

The Sarcomere

In striated muscle the sarcomere is the basic building block of the contractile apparatus. It is composed of a precise geometric arrangement of myosin-containing thick filaments surrounded by an hexagonal array of thin filaments, each of which contains actin and the troponin/tropomyosin regulatory complex (Tobacman 1996).

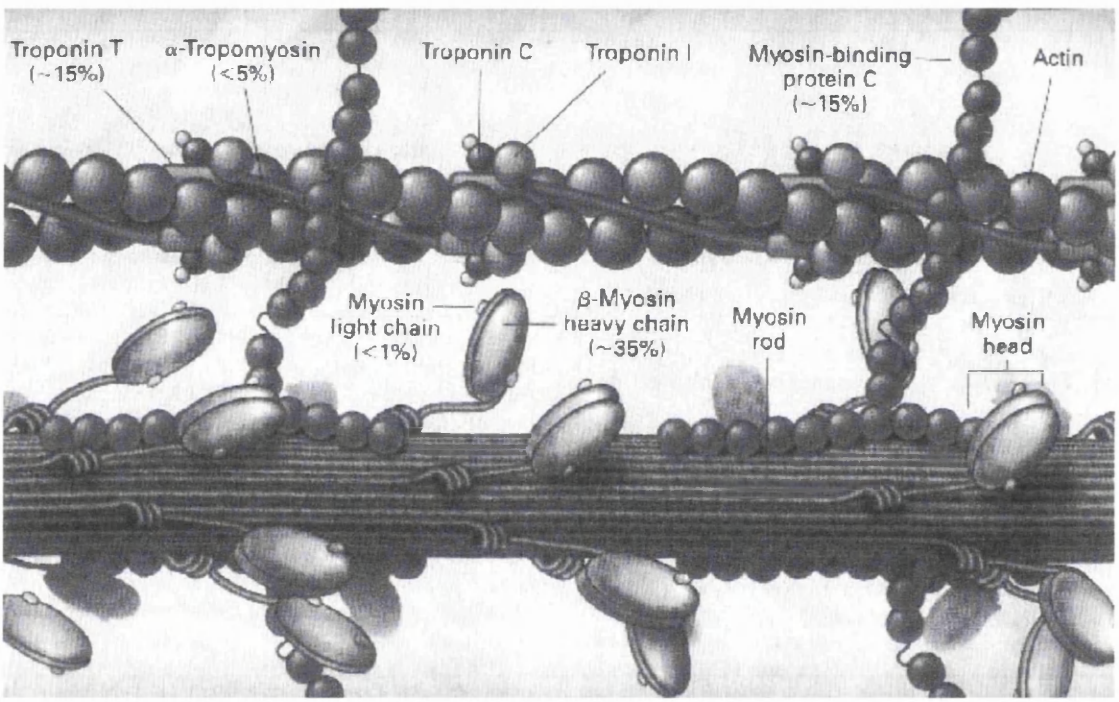


Figure 1.1. Structure of the sarcomere. Adapted from Spirito et al, *NEJM* 1997. 336(11); 775-785

Each thick filament is composed of approximately 300 myosin molecules that are longitudinally stacked. The actin monomers within each thin filament are organised in a double-helical structure with one troponin/tropomyosin complex associated with each repeat of seven actin monomers (Figure 1.1). Each troponin/tropomyosin complex contains four distinct polypeptides: troponin C

(TnC), troponin I (TnI), troponin T (TnT), and tropomyosin (TM). Troponin C is the calcium sensitive component. Troponin I is essential for inhibiting muscle contraction in the absence of calcium by promoting tropomyosin-actin binding (Eaton, Kominz, & Eisenberg 1975). Tropomyosin is a 40 nm long molecule. Its subunits bind together head to tail, forming a continuous chain along the actin thin filament. Each tropomyosin molecule has seven actin binding sites, each binding to an actin monomer in the thin filament. TM lies along the myosin binding sites on actin, preventing contraction. Troponin T can be thought of as the fulcrum through which TnC controls the position of tropomyosin on the surface of the actin filament. Each sarcomere is bound by Z-bands at each end and measures approximately 2.2 μm in length.

The sliding filament theory of contraction is widely accepted (Adelstein & Eisenberg 1980). This defines the geometry and interaction of actin and myosin. The myosin heads are angulated away from the axis of the thick filament. An array of myosin fibres is interdigitated on either side by the thin actin filaments. Myosin heads can specifically interact with binding sites on the actin molecule. By an ATP dependent process a myosin head binds actin and moves it parallel to the fibre axis before releasing it, ready for another cycle. By this means sarcomere length is reduced and in tissue, contraction is seen to take place. Tropomyosin prevents myosin-actin interaction in the resting state by means of steric blocking (Haselgrove 1972).

Myocardial Contractility

Contraction refers to the action whereby a myocardial fibre becomes shorter. A practical definition of *contractility* is the velocity of contraction when other

factors influencing the myocardial oxygen uptake, such as heart rate, preload, and afterload are kept constant. V_{max} is an idealised measure of contractility, defined as the maximal velocity of contraction where there is no load on the isolated muscle or, in the intact heart, no afterload to prevent maximal rates of cardiac ejection. It is extrapolated from a series of measurements of the force-velocity relationship. Where heart rate, preload, and afterload can be kept constant, maximal rate of left ventricular pressure generation (max dP/dt) is used as an index of contractility. This can be obtained by left ventricular catheterisation. In a molecular context, contractility can be thought of in relation to the calcium-contractile protein interaction (Opie 1995).

Excitation-contraction Coupling

Depolarisation of the myocyte membrane occurs through voltage dependent channels. On depolarisation a small amount of calcium enters the cell from the extracellular space. By a process of calcium dependent calcium release from the sarcoplasmic reticulum (ryanodine receptor) the intracellular calcium concentration rises from pCa 7 to pCa 5. The crossbridges undergo cyclic changes, each cycle producing an ATP dependent power stroke that drives the actin filament along the myosin. The tension developed is related to the calcium concentration. Half maximal tension development occurs at a calcium concentration of ~$2x10^{-6}$ M Ca^{2+} (Fabiato & Fabiato 1978). Twofold deviations from the Ca^{2+} midpoint result in at least 90% activation or inhibition in most studies. This corresponds to a Hill coefficient of 3 or more (Babu et al. 1987; Metzger et al. 1993).

An increase in the free calcium ion concentration of the myoplasm enhances the calcium occupancy of troponin C, which by a conformational change causes tropomyosin to move away from the actin binding sites. With the actin binding sites available to the myosin heads the probability of crossbridge attachment increases and contraction occurs. However, in conditions of low intracellular calcium the function of TnI predominates and tropomyosin lies in such a position as to prevent actin-myosin binding. Troponin C can be thought of as the calcium-sensing switch for striated muscle contraction.

In cardiac, as in skeletal muscle, contractile performance is dependent on muscle length. In skeletal muscle, the relationship between sarcomere length and force development is more-or-less bell-shaped, the optimal force being developed at a sarcomere length of about 2.2μm (Gordon, Huxley, & Julian 1966). In heart muscle the sarcomere length rarely exceeds 2.2-2.4μm; connective tissue and titin prevent excessive stretching (Trombitas, Jin, & Granzier 1995). There is evidence to suggest that length-dependent force generation in cardiac muscle is based primarily on length-dependent changes in the interfilamentary separation and crossbridge interaction of actin and myosin rather than the degree of longitudinal overlap (Fuchs & Wang 1996). Both Ca^{2+} sensitivity and Ca^{2+} binding were found to correlate more closely with changes in filament separation than with changes in filament overlap. This may explain the paradox of increasing tension as the degree of myofilament interdigitating overlap decreases with stretch.

Ischaemic Contractile Failure

Contraction is reduced or abolished in ischaemic myocardium (Lee & Allen 1991). In whole animal experiments acute ischaemia causes a marked decline in contractile function within one minute and almost complete contractile failure within 5 minutes (Braunwald & Rutherford 1986; Dobson & Mayer 1973). Perhaps the most striking example of this in clinical practice can be seen during stress echocardiography where regional wall motion abnormalities develop within minutes of ischaemia (Picano et al. 1994). The mechanism of ischaemic contractile failure has been investigated from the biochemistry of contractile proteins *in vitro* to positron emission spectroscopy *in vivo*. Ischaemia is thought to result in hypoxia, reduced delivery of carbohydrate and fatty acid energy sources, depletion of high energy phosphate compounds and an accumulation of potentially harmful low energy phosphates and protons (Dobson & Mayer 1973). Contractile dysfunction is not simply due to an alteration in the calcium transient. Indeed, in early ischaemia the calcium transient may show an increase in peak calcium concentration. Creatine phosphate may be the form in which high-energy phosphates are transported from mitochondria to the sarcomere (Jennings & Reimer 1991). The onset of ischaemic regional contractile dysfunction appears to be better matched temporally to the rapid loss of myocardial creatine phosphate than to that of ATP (Camacho et al. 1988; Hearse 1979). This could explain why total cell ATP concentrations can be high but at the same time ATP availability to the contractile proteins from the creatine phosphate buffer system may be low. With consumption of ATP, inorganic phosphate accumulates and this is associated with a reduction in contractile force for a given concentration of calcium (Korge, Byrd, & Campbell 1993).

Anaerobic metabolism results in intracellular acidosis, a known cause of contractile failure in single cells (Eisner et al. 1989; Poole-Wilson 1978). It is known that intracellular pH in myocytes can fall from 7.0 at rest to 6.2 during acute ischaemia. In an acidic environment the free energy of hydrolysis of the remaining ATP is reduced (Sata et al. 1996). The early phase of contractile failure is temporally associated with an increase in intracellular acidosis and an accumulation of inorganic phosphate. *Early* contractile failure precedes significant changes in the adenosine triphosphate and creatinine buffering systems (Bittl, Balschi, & Ingwall 1987; Marshall 1988). There is increasing evidence that in acidic conditions cardiac TnC is less sensitive to calcium and this may explain the early decoupling of the calcium transient from mechanical contraction (Orchard & Kentish 1990; Metzger et al. 1993).

Response to Ischaemia is Dependent on Contractile Protein Isoform Expression.

If the early decoupling of the calcium transient from mechanical contraction is related to contractile protein pH dependent calcium sensitivity then is there evidence that different isoforms confer differing characteristics? In *adult* dog hearts a reduction in pH from 7.0 to 6.5 induces a 0.4 pCa unit rightward shift in the relationship between pCa and myofibrillar adenosine triphosphatase activity. However, perinatal dog hearts are resistant to the effects of acidosis (Solaro et al. 1986). In neonatal rat ventricular muscle, acidosis caused a significantly smaller fall in tension development when compared with adult rat ventricular muscle (Solaro et al. 1988). Replacement of the slow skeletal TnI present in rat neonatal myofibrils with cardiac TnI restores pH sensitivity

(Martin et al. 1991). In rat hearts, slow skeletal TnI is present for up to 3 weeks after birth (9 months in humans) (Kitsis & Scheuer 1996). There is an increased sensitivity of 5-day-old rabbit hearts to calcium when compared to 22-day-old rabbit hearts. The troponin T isoform differs between the two groups (McAuliffe, Gao, & Solaro 1990). These studies suggest that in adult myocardium, alteration of contractile protein isoforms may affect contractile response to some components of ischaemia. This raises the possibility of manipulating contractile protein isoform expression as a means of attenuating the adverse effects of ischaemia on the force of myocardial contraction.

Structure and Function of TnC

Troponin C has two isoforms, fast sTnC and slow cTnC (Parmacek & Leiden 1991). Both are found in skeletal muscles but only cTnC is found in cardiac muscle. The primary amino-acid sequences are known with sTnC being 160 amino-acids long and cTnC 161 amino-acids long (van Eerd & Takahashi 1975). There is a distinct gene for each isoform (Gahlmann & Kedes 1990; Parmacek & Leiden 1989). The three dimensional structure of sTnC has been defined (Satyshur et al. 1988; Shaw & Sykes 1996). It is a dumbbell shaped molecule 75Å long with the N-domain separated from the C-domain by a 31aa alpha-helix. The C terminal binding sites are non-selective and can bind Ca^{2+} or Mg^{2+} with high affinity (Szczesna et al. 1996). In sTnC two sites in the N-domain have relatively low affinity but bind calcium specifically. These correspond to the sTnC calcium regulatory sites. In cardiac troponin C, site I in the N domain does not bind calcium. Cardiac troponin C contains a single N-terminal Ca^{2+} binding site corresponding to site II in the sTnC molecule

(Rao et al. 1995). According to the model proposed by Herzberg and James, when calcium binds to the N-domain there is a conformational change where helices B and C are thought to move away from A and D and expose a hydrophobic pocket, possibly providing a binding site for TnI (Herzberg, Moult, & James 1986; Gange et al. 1994; Grabarek, Tao, & Gergely 1992). Calcium binding in the N-domain causes a conformational change in the C-domain, reduces the rate of dissociation of Ca^{2+} from, and thereby enhances the affinity for, the C-domain Ca^{2+}-Mg^{2+} sites (Grabarek, Leavis, & Gergely 1986; Wang, Leavis, & Gergely 1983). There is a co-operative effect of the N-domain calcium binding on the C domain interaction with TnT and TnI such that the other subunits move relative to TnC. The tail of TnT, which lies parallel with tropomyosin, moves. Tropomyosin moves relative to the axis of the actin filament exposing the myosin-binding site on the actin molecules (Lehman, Craig, & Vibert 1994). Interestingly, mutants created to test this model were exchanged into skinned fibres and were shown to decrease the Ca^{2+} concentration required for tension activation (da Silva et al. 1993). Calcium occupancy of the specific calcium binding site(s) in TnC strengthens the binding of TnC with TnI, thereby decreasing the negative interaction of TnI with actin/tropomyosin. These findings are consistent with the concept that TnC is the calcium sensitive switch of the troponin trimer, interacting with TnI and TM to allow crossbridge formation between actin and myosin.

Functional Differences Between TnC Isoforms.

The two isoforms of TnC have been shown to have different physiological characteristics. Biochemical experiments with dog cardiac myofibrils found the magnesium-adenosine triphosphatase activity of troponin/tropomyosin-free

myofibrils to be independent of pCa and unaffected by a reduction of pH from 7.0 to 6.5. The affinity of myofibrillar troponin C for calcium decreased as pH was reduced from 7.0 to 6.5 and to 6.2. It was concluded that the mechanism responsible for deactivation of adenosine triphosphatase activity of cardiac myofilaments in acidic pH is related to a reduction in the affinity of myofibrillary troponin C for calcium (Blanchard & Solaro 1984). This finding led to experiments designed to determine the relative sensitivities of different troponin C isoforms to reductions in pH. Myocardial trabeculae from Syrian hamsters had their cTnC chemically extracted and replaced with sTnC (Babu, Scordilis, Sonnenblick, & Gulati1987). In these experiments, permeabilisation of myocardial trabeculae was achieved using Lubrol-WX detergent. Extraction of TnC was achieved by repeated immersion in a rigor solution containing 5mM EDTA and 10mM imidazole at pH7.2. The physiological consequences were investigated by means of conventional pCa-Force and pSr-Force relationships of reconstituted trabeculae. Cardiac TnT is more readily activated by strontium than sTnC. Cardiac muscle reconstituted with sTnC could reach its original level of cardiac tension. The pSr-Force relationship was shifted to the right as in skeletal muscle. Skeletal TnC could confer some physiological properties of skeletal muscle on heart tissue. However, the possibility that sTnC may preserve cardiac contractile function in conditions of acidosis was not tested in this preparation.

The first study to look specifically at the functional differences of sTnC *expression* in myocardial cells was by Metzger (Metzger et al. 1993). It was known that depressed contractility secondary to acidosis was more marked in cardiac muscle than in skeletal muscle. Therefore a transgenic mouse expressing sTnC in myocardium under a α-myosin heavy chain promoter was

produced. Of three independent lines produced, only one was investigated. Although northern blot analysis was used to prove gene expression, the possibility of functional change caused by insertional mutagenesis was not excluded. Single cells were selected for investigation only if they did not contract when exposed to a pSr of 5.0, indicating a relative predominance of sTnC in their response. The ratio of cTnC:sTnC in these single cells was not specified. Nevertheless in conditions of acidosis, as may occur in ischaemic myocardium, there were significant differences between transgenic single myocytes and controls. Tension-pCa curves were plotted for both groups. When pH fell from 7.0 to 6.2 the change in pCa_{50} (reduction in calcium sensitivity) was significantly less in the transgenic cells expressing sTnC than in controls. The authors concluded that sTnC could remain functional in conditions of acidosis that significantly impair cTnC.

Using skinned psoas muscle it was demonstrated that replacing cTnC in slow fibres with fast sTnC decreased pH sensitivity. The reciprocal result was found in exchanging sTnC for cTnC in fast twitch fibres (Metzger 1996). Interestingly, four animals were used as a control group to show that the extraction process did not have a significant effect on baseline calcium sensitivity. It should be noted that extraction of the endogenous TnC was of the order of 70% and that this data represents a mixture of the isoforms in fibres. The altered group of n=8 was compared against a notional control of zero change rather than against the actual control group. The effect on diastolic function was not specified. Skeletal TnC did not improve calcium responsiveness of myocytes at pH 7.0. Its potential to act as a calcium sensitiser in ischaemic myocardium was not investigated. In another study employing skinned psoas muscle preparation, an ^{88}KGKSEEE^{94} deleted sTnC

mutant showed higher calcium sensitivity than normal sTnC. The effect of acidosis was not tested (Gulati et al. 1992). It is not known if expressing this mutant gene in whole heart preparations would have a positive inotropic effect. From the transgenic studies of Metzger and the TnC replacement studies in isolated muscle, it would appear that TnC isoform has a significant influence on muscle contractile response to acidosis. Of particular importance is that sTnC expression in cardiac muscle could confer a potentially beneficial resistance to acidosis *in vivo.* It has not been determined what effects other components of ischaemia may have on such tissue *in vivo.*

The Interactions with Other Subunits

Troponin I Isoforms

When cellular calcium levels are low, TnI interacts with and inhibits actin-myosin crossbridge formation. Cardiac TnI differs from skeletal TnI in that it has a 27-33 amino-acid long N-terminal extension which when phosphorylated results in a lowering of the TnC regulatory site II affinity for calcium (Guo et al. 1994). Phosphorylation of cardiac Troponin I increases the rate of cardiac muscle relaxation and reduces calcium sensitivity (Dohet et al. 1995; Zhang et al. 1995).

Troponin T Isoforms

Rabbit ventricular muscle can express at least four troponin T isoforms. Their distribution changes with development. There is some evidence to suggest

that the pCa-force characteristics of rabbit myocardium can be affected by the troponin T isoform (Nassar et al. 1991). TnT has no known calcium binding or phosporylation regulatory sites. Ischaemic injury can change the immunoreactivity of troponin T. This may be as a result of calcium-dependent activation of both calpain proteolysis and transglutaminase cross-linking (Gorza et al. 1996).

Myosin Isoforms

Transgenic Mice expressing fast muscle-specific myosin light chain in the ventricle showed reduced left ventricular contractility and relaxation (Gulick et al. 1997).

Regulation of Contractility

Calcium release and uptake is closely regulated by G protein-coupled beta-adrenergic receptors through the action of the second messenger cAMP. An increase in cAMP level leads to phosphorylation of key regulatory proteins affecting intracellular Ca^{2+} homeostasis. In particular, phospholamban phosphorylation decreases its inhibitory effect on the sarcoplasmic reticulum ATPase (SERCA). This results in SERCA pumping more calcium into the SR during diastole and an increase in SR calcium release during subsequent systole. The increased calcium release is associated with an increased force of contraction. Myosin phosphorylation enhances the calcium responsiveness of the cardiac contractile proteins (Morano et al. 1985). Myosin phosphorylation is increased by cAMP in response to beta-adrenergic

stimulation. This may explain why adrenaline increases the rate of crossbridge cycling independently of the degree of activation of contractile proteins by calcium (Saeki et al. 1990). In the heart the predominant adrenergic receptor is the beta1-adrenergic receptor. The beta-adrenergic receptors themselves are regulated by a set of specific kinases, termed the G-protein-coupled receptor kinases (Ben-Yehuda & Rockman 1996). In an interesting series of experiments, transgenic mice over-expressing the human beta2-adrenergic receptor showed enhanced contractility as measured by an elevated LV dP/dt_{max} (Miliano, Allen, & Rockman 1994).

Calcium Sensitising Drugs Show Promise.

There has been interest in compounds which sensitise the contractile apparatus to the effect of calcium. For a given calcium transient, such compounds would have the potential to increase force of contraction and thereby act as positive inotropes. Theoretically, a calcium sensitising compound may have a neutral effect on contractility but be associated with comparatively less calcium shuttling and consequently less oxygen consumption. Inotropes which increase intra-cellular calcium have been associated with premature death in patients with heart failure (Packer et al. 1991). A positive inotrope, which reduced oxygen consumption without increasing intracellular calcium shuttling, would be an attractive treatment in both heart failure and ischaemic heart disease. In a rat model of endotoxic shock, the sensitivity of cardiac contractile proteins to calcium is decreased. This abnormality was reported to be reversed by the calcium sensitising drug MCI-154 (Ming et al. 2000). MCI-154 has been shown to increase cardiac output whilst decreasing relative myocardial oxygen consumption in patients with coronary artery disease. However, in patients with chronic heart failure, it had a neutral effect with no symptomatic or mortality effect (Takaoka et al. 1997).

Levosimendan binds to cTnC and can improve both systolic and diastolic contractile function (Sorsa et al. 2000). Levosimendan exhibits inotropic effects as a calcium sensitiser. Levosimendan has been tested in two clinical trials the preliminary results of which have been reported in abstract form. It has been employed in patients with low-output failure (LIDO, or Efficacy and Safety of Intravenous Levosimendan in Severe Low-Output Heart Failure,

trial) and patients with heart failure following myocardial infarction (RUSSLAN, or Randomized Study on Safety and Effectiveness of Levosimedan in Patients with Left Ventricular Dysfunction after an Acute Myocardial Infarction, trial). In the LIDO trial, levosimendan was compared with dobutamine during a 24-hour treatment period in 203 patients with low cardiac output and decompensated heart failure (Follath F et al. 1999). The primary end point was defined as the proportion of patients in each treatment group showing improved haemodynamic response at 24 hours and no need for other "rescue" therapy. The preliminary results of this study indicate that levosimendan was more effective than dobutamine in achieving the primary end point (28% vs. 15%). Surprisingly, after 6 months, mortality for patients receiving dobutamine was 38%, compared with 27% for those treated with levosimendan. Levosimendan also resulted in more out-of-hospital days at 1-month follow-up. Levosimendan has an energetically favourable short-term profile in the treatment of congestive heart failure. It enhances cardiac output without oxygen wasting, particularly by improving efficiency in the right ventricle (Ukkonen et al. 2000). The RUSSLAN trial evaluated the effects of levosimendan versus placebo in 504 patients with decompensated heart failure following acute myocardial infarction (Moiseyev et al. 1999). Patients were evaluated for up to 14 days following therapy. The study results revealed a significant reduction in the combined risk of death and worsening failure during the first 24 hours following randomisation (P =0.044). All-cause mortality after 14 days was also significantly lower in the levosimendan-treated patients (11.4% vs. 19.6%, P =0.029). Patients treated with a higher dose of levosimendan (0.4 μg/kg/min) showed a trend toward higher incidence of ischaemia and hypotension. Calcium sensitisers may generate force with smaller amounts of Ca^{2+} by increasing the responsiveness of myofilaments to

Ca^{2+}. They have the potential to cause diastolic dysfunction but whether this will be clinically significant remains to be determined. Pure calcium sensitisers may be inotropic without being arrhythmogenic (Kawai, Lee, & Orchard 2000).

Troponin C as a Therapeutic Candidate

Troponin C is an attractive candidate for therapeutic intervention. When myocardium becomes ischaemic there is rapid reduction in intracellular pH. The function of cardiac troponin C is significantly affected by intracellular acidosis and is in part responsible for the early decoupling of the calcium transient and contractile dysfunction. In comparison sTnC is relatively unaffected by moderate degrees of intracellular acidosis. By displacing a proportion of the cardiac troponin C with the skeletal isoform, there would be the possibility of rendering cardiac muscle relatively resistant to the effects of intracellular acidosis. Perhaps such altered myocardium would be able to contract for longer in conditions of ischaemia. There is however, a possibility of a relative increase in calcium sensitivity at lower pH leading to adverse effects on myocardial relaxation. Other limiting factors may be a further decrease in intracellular pH, an increase in free inorganic phosphate, a change in intracellular calcium homeostasis and possibly a pro-arrhythmic effect.

Troponin C isoform *mutants* could be used as calcium sensitising agents, thereby increasing the force of contraction for a given amount of calcium released by the sarcoplasmic reticulum (Gulati, Babu, Su, & Gulati 1992). Perhaps more importantly, such calcium-sensitising troponin mutants may

result in a normal force of contraction but a reduction of SR calcium release during each calcium transient. A reduction in the calcium released from the sarcoplasmic reticulum during the calcium transient would be expected to reduce the energy required to pump calcium back into the sarcoplasmic reticulum. This would have the potential of maintaining normal cardiac function but reducing the energy require for each contraction, increasing contractile efficiency and reducing oxygen demand.

Other Potential Therapeutic targets

Sarcoplasmic Reticulum Calcium ATPase.

In heart failure, there is abnormal calcium handling within myocytes consistent with a reduction in SERCA activity. SERCA function is modulated by phospholamban. In its dephosphorylated state, phospholamban inhibits SERCA function. In heart failure, the apparent reduction in SERCA activity is a result of a down regulation in SERCA expression and a relative increase in dephosphorylated phospholamban. This imbalance could be improved by either increasing SERCA expression or increasing the proportion of phospholamban in its phosphorylated state (Chossat et al. 2001). This hypothesis has been tested in myocytes isolated from patients with end stage heart failure. An adenoviral vector was employed to cause over-expression of the gene for SERCA2a. Improvements were found in parameters of both contraction and relaxation (del Monte et al. 1999). Similar desirable effects were found in the whole heart physiology of aging rats (Schmidt et al. 2000).

Phospholamban

In its dephosphorylated state, phospholamban modulates sarcoplasmic reticulum calcium sequestration by inhibiting SERCA (Davia et al. 1999). Increasing expression of phospholamban is likely to have detrimental effects on contractile function and has been found to result in premature death in transgenic mice (Dash et al. 2001). This finding may be explained by enhancement of adrenergic tone. Over expression of a phospholamban mutant which acts physiologically as a phosphorylated wild type molecule would have the potential to enhance the function of endogenously expressed SERCA. The phospholamban Ser(16) amino-acid residue has been identified as a potential target (Chu et al. 2000).

Apoptosis and Bcl-xL

In severe heart failure, there is evidence of myocytes loss secondary to programmed cell death (apoptosis). Bcl-xL is an anti-apopototic molecule which if up-regulated would have the potential to reduce the loss of viable cardiac myocytes by apoptosis and may slow the progression of heart failure (Bartling et al. 1999; Latif et al. 2000; Milting et al. 1999)

A downstream signalling molecule of insulin-like growth factor-1 (IGF-1) has been shown to reduce the rate of apoptosis in isolated cardiac myocytes.

Expression of activated forms of either PI 3-kinase or Akt in cardiomyocytes was sufficient to inhibit hypoxia-induced apoptosis *in vitro* (Matsui et al. 1999).

Vascular Endothelial Growth Factor (VEGF)

There is increasing interest in using Vascular Endothelial Growth Factor (VEGF) to improve the blood supply of ischaemic myocardium which is unsuitable for either PTCA or coronary artery bypass grafting (Byun et al. 2001; Su, Lu, & Kan 2000; Tanaka et al. 2000). Unfortunately at present there is no known pharmacological method for increasing endogenous production of VEGF. Attempts have been made to infuse the gene product, but the most impressive results have been with gene transfer methods. In a small pilot study of only five patients, plasmid DNA encoding VEGF (phVEGF165) was introduced into ischaemic myocardium by direct needle injection at mini-thoracotomy. These patients had been selected because they remained severely limited by angina and were unsuitable for conventional treatment either by PTCA or coronary artery bypass surgery. In this group, objective evidence of ischaemia was documented using dobutamine single photon emission computed tomography (SPECT)-sestamibi imaging in all patients. More importantly, all patients had a significant improvement in their anginal symptoms (Losordo et al. 1998). This led to a phase one clinical trial in 20 patients with two different doses of plasmid DNA (Symes et al. 1999). In this group, all patients had class III or IV angina. Similarly impressive results were achieved with significant symptomatic improvement in patients with "inoperable" coronary artery disease. An adenoviral vector encoding a different vascular endothelial growth factor (VEGF121) has been used as sole therapy in a group of six patients with angina (Rosengart et al. 1999).

Treadmill exercise assessment suggested improvement in most individuals. However, no long-term safety data are available for any of these studies and clinical trials are ongoing. It is important to note that these small studies were open label studies and it is possible that a placebo effect may have contributed to the subjective improvement in some patients. A blinded randomised study would be required to exclude this possibility. Other growth factors with potential to improve the vascularisation of ischaemic myocardium are under investigation. In a pig model of stress-induced myocardial ischaemia, intracoronary injection of a recombinant adenovirus expressing human fibroblast growth factor-5 (FGF-5) was associated with improved blood flow and histological evidence of angiogenesis (Giordano et al. 1996). Others have been investigating the potential of FGF-1 (Schumacher et al. 1998).

Transforming Fibroblasts into Striated Muscle Cells.

Following a myocardial infarction, an akinetic scar containing non-contractile fibroblasts replaces the dead myocytes. There is interest in molecular methods of transforming these fibroblasts into contractile cells and possibly improving ventricular systolic function. The gene *MyoD* plays a role in establishing and/or maintaining the differential phenotype of adult fast skeletal muscle fibres (Metzger, Rudnick, & Westfall 1995). An adenovirus expressing *MyoD* can cause cultured cardiac fibroblasts to express contractile proteins (Murry et al. 1996). Although embryonic skeletal MHC expression has been achieved in an *in vivo* model, myogenic conversion of infarcted myocardium has not yet been shown. In clinical practice, transformation of a proportion of fibroblasts into contractile cells *in vivo* would be of little benefit if they remain

electrically isolated from normal myocardium by surrounding fibroblasts. There would remain the difficulty of co-ordinating contraction of these cells with the contraction of healthy myocardium. Should the electrical coupling become possible, there would be the risk of arrhythmogenesis occurring at the interface of slow and fast conduction.

Gene Therapy for Arrhythmias

Genetic manipulation of cardiac excitability may be possible (Johns et al. 1995; Lawrence et al. 1995). In a canine model of heart failure, single cells show altered electrical properties when exposed to an adenovirus expressing potassium channels (Nuss et al. 1996). The exposed myocytes showed shortening of the action potential duration. Some arrhythmic deaths are the result of single gene defects (Baroudi & Chahine 2000; Schwartz et al. 2001). The possibility of correcting such defects by gene transfer methods is attractive. However, methods such as direct needle injection as used in the VEGF studies would be inappropriate in this situation. Correction of a small part of the myocardium may lead to heterogeneity of regional refractory periods. Regional differences in the refractory period may lead to re-entry circuits with the potential for a pro-arrhythmic effect. To avoid this problem, a method of homogeneous gene transfer would be required.

Antisense DNA

Antisense DNA techniques, through a number of mechanisms, can effectively down regulate the expression of target proteins. It is possible to design a

sequence of DNA that is complementary to the messenger RNA of an unwanted gene. On expression of this DNA sequence, the target gene expression is blocked at the level of mRNA. One potential application for this technology is in coronary artery re-stenosis following PTCA. It has been demonstrated that antisense c-*myc* oligonucleotides can inhibit arterial smooth muscle cell accumulation in a rat carotid artery injury model (Bennett et al. 1994; Simons et al. 1992). More recently, antisense DNA methods have been used in a rat model of hypertension to reduce expression of cardiac beta1-adrenegic receptors (Zhang et al. 2000). The result was a significant anti-hypertensive effect that persisted for more than 18 days.

If a large number of candidate genes are to be tested for their potential as therapeutic targets then efficient *in vitro* and *in vivo* methods of gene transfer and control will be required. Methods of controlling gene expression in tissues ranging from single cells to whole organs need to be developed. Genetically manipulated mice have been used to over-express and under-express genes. The development of transgenic mouse lines is both time-consuming and expensive. There are established models of rhythm disturbance and heart failure in rabbits and dogs. These animals have a longer gestation period than mice and are therefore unattractive candidates for the production of transgenic lines. There is a need for gene transfer methods to such animals. Such methods are likely to prove a powerful tool in the evaluation of potential therapeutic targets.

Gene Transfer Methods

Introduction

To investigate the potential physiological benefits of controlling target gene expression, methods of introducing genes of interest into cells are required. The perfect vector for this purpose would be simple and inexpensive to construct. It could accommodate large DNA sequences. It would transfect cells efficiently both *in vitro* and *in vivo*. It would have a low potential of provoking a host immune response and would integrate the desired DNA sequence into the host genome in a precise manner. Long-term and tissue specific gene expression would also need to be possible. Unfortunately, such a vector does not yet exist.

Plasmid DNA

Direct needle injection of plasmid DNA into muscle results in up-take of a proportion of the DNA into a small number of surrounding cells, including myocytes. This is true both for skeletal muscle and cardiac muscle. DNA is absorbed into typically 100-200 surrounding cells by a process of endocytosis. The DNA remains epichromosomal and the duration of expression of foreign gene is about 2-3 months. Plasmid DNA is easily produced using bacterial cultures. Commercially available plasmids make incorporation and manipulation of DNA sequences inexpensive. Direct needle injection of plasmid DNA is suitable for large and small animals alike. However, direct

needle injection of DNA is an inefficient means of delivering genes of interest and invariably results in tissue damage along the needle track. Nevertheless, it is a method which has been used to study the characteristics of gene promoters for their tissue specificity and hypoxic responsiveness, and remains a valuable tool for this purpose (Prentice et al. 1997). In a gene therapy context, it has potential where the gene of interest is a chemo-tactic molecule such as in VEGF or is hormonal such as erythropoeitin. However, direct injection of plasmid DNA would not be appropriate where the application required homogenous gene expression throughout an entire organ (Acsadi et al. 1991; Prentice et al. 1997; Wolff et al. 1992).

Replication Deficient Adenoviral Vectors.

Adenoviral vector mediated gene transfer to the heart was first demonstrated by Stratford-Perricaudet. Replication deficient adenoviral vector encoding beta-galactosidase was injected intravenously. Gene product became detectable in a small proportion of myocytes (Stratford-Perricaudet et al. 1992). Adenoviruses are a family of viruses (over 40 serotypes) that cause benign respiratory tract infections in humans. A benign clinical profile is expected, based on their long-term use as a vaccine for adenovirus respiratory tract infections by the US Army (Takafuji, Gaydos, & Allen 1997). Adenoviruses are non-enveloped icosahedral double-stranded DNA viruses. They gain entry into cells by receptor-mediated endocytosis via the coxsackie receptor (Roelvink et al. 1998). Adenoviral vectors which can gain entry to cells without the need for a coxsackie receptor are under development. These may have an application in cancer gene therapy where target cells may not express coxsackie receptors (Krasnykh et al. 2000).

The pathological effect of adenoviruses is due to the host mediated inflammatory responses and to the lysis of infected cells as viral replication proceeds. Viral replication takes place in the nucleus of the host cell without integration into the host DNA (Schneider & French 1993). The adenoviral genome is about 36Kb long with the E1 region being essential for viral replication. Replication deficient viruses can be produced by replacing the E1 region of the viral genome with a gene of interest (Hitt et al. 1995; Wilson 1996). The virus is then amplified in a permissive E1-expressing human kidney 293 cell line. The resulting vector is capable of infecting a broad range of cell types. However, in the absence of E1 gene products, the virus cannot replicate. Theoretically, such a replication deficient virus would not cause lysis of infected cells. Nevertheless, the viral capsid and other viral gene products have the potential to provoke an immune response. As wild-type viruses are ubiquitous, the possibility of recombination of wild type virus with replication deficient vectors exists. There is a possibility of a foreign gene of interest "escaping" from a replication deficient virus into a replication competent wild-type virus through a process of homologous recombination. Interestingly, some cells which have been infected with the Ebstein-Barr virus have been found capable of sustaining chronic infection of both replication competent and replication incompetent adenoviruses (Horvath et al 1991).

E1 deleted adenoviral vectors are limited to an insert capacity of 8Kb. An adenoviral vector has been developed which has almost all viral coding sequences removed (E1,E2,E3 and E4) and can carry an expression cassette of more than 28Kb (Kochanek et al. 1996).

Duration of Reporter Gene Expression

Although long-term gene expression in heart muscle was reported following simple intravenous injection of adenovirus (Stratford-Perricaudet, Makeh, Perricaudet, & Briand. 1992), this has not been found in subsequent experiments involving arterial injections. In direct-injection or catheter-mediated delivery into heart muscle, expression was detectable within 3 days (Guzman et al. 1993; Li et al. 1995). However, by day 21 not only was the reporter gene beta-galactosidase not detectable but neither was the reporter gene cDNA. In adenovirus-mediated gene transfer the adenoviral DNA remains epichromosomal and would be vulnerable to intracellular mechanisms of DNA clearance. However, a more likely explanation for transient gene expression following adenoviral-mediated gene transfer is an inflammatory response. Both humoral and cell-mediated responses have been described. Pre-immunisation of rats to a beta-galactosidase expressing adenovirus reduced the expression duration of the reporter gene from 21 days to 6 days. However, neonatal intrathymic injection of vector induced long-term beta-galactosidase expression for more than 2 months after direct ventricular injection (Gilgenkrantz et al. 1995). Intrathymic injection in early development could allow the host immune system to recognise the adenoviral vector antigens as self. The finding of Gilgenkrantz et al supports the hypothesis that the host immune system can limit duration of foreign gene expression following adenoviral vector mediated gene transfer. These studies suggest that the immune response to adenoviral-infected cells is at least in part antibody mediated. There is strong evidence to suggest that the *early* inflammatory response to cells infected by adenovirus is cell-mediated (Yang et al. 1994). Both adenovirus-specific proliferative CD4+ T cells and CD8+

cells are implicated (Flomenberg et al. 1995; Flomenberg et al. 1996). Host immunosuppression prolongs the therapeutic effect of adenovirus-mediated gene expression (Fang et al. 1995).

Although adenoviral vectors deleted for all viral coding sequences result in extended expression of their transgenes, the E3 region of wild type adenovirus has been implicated in evading host defences and may be a desirable component of an adenoviral vector (Bennett et al. 1999; Gooding et al. 1988; Morsy et al. 1998). Gene Products encoded in the E3 region can interfere with MHC expression within infected cells and reduce the potential for a cell-mediated inflammatory response. The presence of the E3 region has been found to render infected cells less susceptible to the effects of TNF-alpha (Gooding et al. 1988). The mechanism for this effect is poorly understood.

Efficiency of Adenoviral Vectors

Adenoviral vectors infect cells by a receptor-mediated process. The efficiency of adenoviral mediated gene transfer is at least one order of magnitude greater than that for naked plasmid DNA (Guzman et al. 1993; Kass-Eisler et al. 1993).

Cationic Liposomes

Cationic liposomes are positively charged artificial lipid vesicles that incorporate negatively charged DNA and deliver nucleic acid to cells through fusion with cell membranes. They are more efficient at transfecting cells than naked plasmid DNA. However, they are inefficient when compared with adenoviral vectors but have the advantage that they are not thought to be immunogenic in themselves. Cationic liposomes have been used in preliminary clinical studies of gene therapy in cystic fibrosis (Caplen et al. 1995). In dogs, gene transfer to the coronary circulation has been achieved using a porous perfusion balloon catheter system in the coronary artery (Chapman et al. 1992). Cationic liposomes have been used to introduce foreign genes to rabbit myocardium via the coronary circulation. By this route they have been associated with micro-infarction (Wright et al. 1998). Cationic liposomes have been successfully employed in gene transfer to peripheral arteries (Takeshita et al. 1994).

Vaccinia Vectors (Pox viruses)

Pox viruses have been used extensively in smallpox vaccination. They are 186 Kb DNA viruses with a double envelope. Their DNA remains in the cytoplasm and the risk of insertional mutagenesis is thought to be low. Poxviruses have not been successfully used to achieve foreign gene expression in myocardium but have mainly been used in cancer gene therapy (Peplinski, Tsung, & Norton 1998).

Retroviral Vectors.

The first retroviral gene-transfer vector was described in 1981 (Wei, Gibson, & Spear 1981) and the first patient treated (for adenosine deaminase deficiency) was in 1990 (Anderson, Blaese, & Culver 1990). Retroviruses are diploid positive-stranded RNA viruses packaged in a glycoprotein envelope. Using reverse transcriptase the RNA is transcribed into DNA, which is pseudo-randomly integrated into the host genome. The normal cellular machinery is then utilised to make proteins as encoded by the virus. Ischaemic/reperfused myocardium has been shown to express recombinant protein following direct needle-injection of retroviral vector (Prentice et al. 1996). Gene transfer to the arterial wall by retrovirus has resulted in gene expression for more than 5 months without the need for immunosuppression (Nabel, Plautz, & Nabel 1990). However, compared with adenoviral vectors, infection efficiency is low and it is more difficult to produce high titre vector (Flugelman et al. 1992). However, for genomic integration the target cells must divide. This limits their use in the myocardium as cardiomyocytes are terminally differentiated and do not undergo mitosis (Miller, Adam, & Miller 1990). This is true for first generation murine leukaemia viruses. However, lentiviruses do not require cell division for genetic integration (Goldman et al. 1997). Insertional mutagenesis of the viral DNA has the potential to cause a tumorigenic event and safety concerns have been raised. A retroviral vector has been successfully employed to improve the lipid profile of patents with homozygous familial hypercholesterolaemia by corrective gene transfer to hepatocytes (Grossman et al. 1995).

Herpes Virus Vectors

Herpes virus vectors are large enveloped double-stranded DNA vectors that are capable of infecting myocytes *in vivo* (Coffin et al. 1996, Wright et al. 2001). They can accommodate very large foreign DNA fragments and can be rendered replication deficient by deletion of immediate early genes (typically deletion of ICP27). In infected cells, the episomal viral DNA structure remains stable and there is the potential for long-term gene expression. However, it is difficult to produce herpes vector with a titre of more than 10^9 infective particles per millilitre. Adenoviral vectors can be propagated to titres of 10^{13} pfu/ml and for this reason have been favoured over herpes vectors.

Adeno-associated Viral Vectors.

Adeno-associated virus (AAV) is a 4.7 Kb long single stranded DNA parvovirus. It integrates into the host genome and shows a predilection for a specific site in chromosome 19. Integration into the host genome can be in a site-specific manner (Russell & Hirata 1998). Perhaps the greatest advantage of AAVs over adenoviral vectors is that they can efficiently transduce muscle fibres *in vivo* without activation of cellular of humoral immunity (Jooss et al. 1998). No viral genes are expressed and they have the potential to cause long-term foreign gene expression (Kaplitt et al. 1996, Lynch et al. 1997). The main disadvantage of AAVs when compared with adenoviral vectors is the difficulty of producing AAVs at high titre. Systems to produce adeno-associated viruses have required potentially contaminating helper adenovirus.

Methods of improving production efficiency are under investigation (Maxwell, Harrison, & Maxwell 1997). As production methods improve, adeno-associated viruses are likely to replace adenovirus vectors both as investigative tools and for gene therapy applications

Routes of Adenoviral Administration.

Intravenous injection of replication deficient adenoviral vector by Stratford-Perricaudet showed that myocardium could take up adenovirus. This is an inefficient route when attempting to infect heart tissue as the highest uptake was found in the liver and only 0.2% of myocytes expressed the reporter gene. Efficiency of gene transfer was greatly improved by selective coronary injection. In 1993 Barr *et al* injected 10^9-10^{10} plaque forming units (pfu) of adenoviral vector using a selective coronary catheter. They reported peri-vascular gene transfer and found reporter gene expression in 10%-30% of myocytes within the distribution of the injected artery. A co-author has since reported that infection of 60% of myocytes has been achieved (Leinwand & Leiden 1996). However, these findings have proved difficult to reproduce. Indeed, in the six years that followed the original report, no group reported any change of myocardial function following gene transfer by this method.

Methods of Gene Transfer to Myocardial Tissue.

Important physiological information can be obtained from investigation of single cardiac myocytes. Infection and investigation of single myocytes would be sufficient to answer important questions for some genes of interest.

However, transfer to whole organs and investigation of intact live animals may be required for other genes. Thus, a variety of gene transfer methods will be required.

Cultured Cells

Adult myocytes are difficult to isolate and maintain in culture for gene expression studies. They de-differentiate in long-term culture. Adenoviral vectors can infect cultured myocytes with high efficiency (Krishenbaum et al. 1993). Potentiation of β-adrenergic signalling by adenoviral-mediated gene transfer has been demonstrated in cultured adult rabbit ventricular myocytes (Drazner et al. 1997). Interestingly, adenoviral infection of myocardial cells *per se* has been reported to induce an enhanced sensitivity to β-adrenergic agonists by increasing the concentration of the stimulatory G-protein (Novotny et al. 1994). Cultured cells can be reliably transfected using a relatively small amount of virus. Single cells can be used to investigate intra-cellular secondary messenger signalling. Investigation of the electro-physiological characteristics of single cells is possible by means of patch clamping. However, it is difficult to measure the force of contraction in single cells. Studies of the contractile properties of single cardiac myocytes have largely been limited to measuring frequency and velocity of contraction and relaxation in unloaded systems.

Cultured Trabeculae

Trabeculae have the advantage over single cells in that they can be readily manipulated and attached to force transducers. Small trabeculae can be maintained in Ringer solution for extended periods. These preparations are suited to the investigation of contractile physiology. The intracellular environment of such preparations can be controlled by chemically skinning the tissues. By permeabilising the cell membranes, the bathing solution surrounding the preparation becomes an extension of the intracellular environment. This allows control of all intracellular ions and energy sources. Alterations in pH, potassium, lactate, inorganic phosphate and creatine buffer can be made to simulate components of ischaemia. The main disadvantage of this preparation over a whole heart preparation is that ischaemia is mimicked and true ischaemia, either supply or demand, is not possible. Isolated trabeculae have been transfected with adenoviral vector and it has proved possible to keep such tissue viable for up to 3 days following transfection (Janssen et al. 1998).

Isolated Whole Heart Preparations

Ex vivo whole heart physiology can be investigated in Langendorff perfusion models. Typically, the whole heart is rapidly removed from a euthanised animal and retrogradely perfused by a cannula placed in the ascending aorta. In a working heart preparation, control of pre-load, after-load and after-load compliance is possible. External pacing can control both atrial and ventricular

rate. Components of ischaemia can be mimicked by alteration in the constituents of the perfusate. Restricting or occluding coronary flow using a coronary artery snare can produce true ischaemia. Measurement of force of contraction, cardiac output and regional EMG is possible. A Langendorff apparatus has been used to transfect rabbit myocardium by re-circulating adenoviral vector in a limited volume of perfusate. Donahue et al reported highly effective gene transfer to rabbit heart and identified critical parameters influencing the efficiency of adenoviral gene transfer. These included the use of crystalloid solution as opposed to whole blood; high coronary flow rate; exposure time; virus concentration, and temperature. More recently, the same group found that decreasing perfusate calcium concentration, or pretreating with serotonin or bradykinin, significantly decreased the exposure time necessary to achieve widespread infection (Donahue et al. 1997; Donahue et al. 1998). The disadvantage of such an approach is that it can take several days for foreign gene expression to become detectable. Conventional isolated preparations will not continue to contract for several days. One group have attempted to over-come this shortcoming by transplanting transfected hearts into recipient animals for a period to allow gene expression prior to further investigations (Pellegrini et al. 2000).

Intra-coronary Injection

Using $1x10^{10}$ pfu/ml of adenovirus injected into the left coronary artery of 4Kg New Zealand white rabbits, 30% of myocytes in the distribution of the coronary artery have been shown to express reporter gene (Barr et al. 1994). Adenoviral vectors have the potential to induce an inflammatory response. In keeping with previous studies no reporter gene expression was evident at, or after, day 21. Even though histology was taken at 5 days, 2 weeks, 1 month and 2 months, no inflammatory response was noted in any of the hearts following intra-arterial adenovirus infusion. This finding remains unexplained. In minipigs intracoronary adenoviral vector was delivered at thoracotomy using a needle in the circumflex artery. Despite $2x10^{10}$ pfu of virus being injected, only low-level gene expression was observed in the myocardium (Muhlhauser et al. 1996). This was in contrast to the very high levels of gene expression found in rabbit. The attraction of the intracoronary injection method is that it is minimally invasive and animals can recover, allowing gene expression to occur before physiological investigation.

Direct Needle Injection into the Heart.

Direct injection of adenoviral vector into the myocardium results in high levels of reporter gene expression when compared with naked plasmid and can be achieved in both large and small animal models (Gal et al. 1993; Guzman et al. 1993; Lin et al. 1990; Magovern et al. 1996). Unfortunately, tissue damage occurs along the needle track, but the extent of inflammation induced by

adenoviral injection is not significantly different from that induced by injection of saline (Li et al. 1995). When direct injection of adenoviral vector was performed in models of myocardial infarction, transgene expression was restricted to residual cardiomyoctes in the periphery of the infarct area (Leor et al. 1996).

Injection of adenoviral vector into the ventricular cavity results in gene expression particularly in the heart, intercostal muscles, and thymus. Intravenous injection causes significant expression in the liver but is an inefficient method of gene transfer to the heart (Huard et al. 1995). It is possible to restrict reporter gene expression to the heart by use of a ventricle-specific-myosin light chain-2 promoter (Rothmann et al. 1996). Although this may result in lower levels of expression than can be achieved using a constitutive viral promoter, this may be adequate for application such as revascularisation by VEGF expression (Buttrick et al. 1992). For homogenous gene transfer to whole hearts a more efficient method of gene transfer would be required.

Aortic and Pulmonary Cross-clamp Method

In this approach, a catheter is inserted in the left ventricular apex and advanced beyond the aortic valve. A high-concentration adenoviral preparation is then injected through the catheter while the aorta and pulmonary artery are cross-clamped distal to the catheter tip for a period of 10 to 40 seconds. In rats this method has achieved grossly homogeneous transduction of cardiac myocytes throughout the left and right ventricles of the heart (Hajjar et al. 1998). More importantly, this technique can produce

dramatic, transgene-specific physiological effects on ventricular function *in vivo*. The success of this approach likely reflects *in vivo* optimisation of the parameters previously shown to be important for *ex vivo* gene transfer, as well as high-perfusion pressure that presumably allows the opening of capillaries and optimises the myocardial area of virus exposure (Donahue et al. 1997). By cross-clamping both the pulmonary artery and the aorta, blood return to the left ventricle is minimal, and the left ventricular end-diastolic pressure does not increase. This allows perfusion of the coronary circulation at relatively low downstream pressure, and the endocardium can be efficiently infected. Correlates of this method in humans have not yet been established and a minimally invasive catheter based method of homogenous gene transfer would be preferable.(Hajjar et al. 1998)

Retro-infusion

It has proved possible to transfect myocardium using adenoviral vectors retro-infused down the coronary veins. This method has potential for creating homogeneous transgene expression in an intact organ but may prove difficult where animals are required to recover.
(Boeckstegers et al. 2000)

Pericardial

Transfection of a thin epicardial layer of heart cells is possible by injection of adenoviral vector into the pericardial space. As in transduction of single cells in culture, this method has the advantage of long exposure times and requires only a small amount of virus (Fromes et al. 1999).

Gene Transfer to Vessels.

In vivo gene transfer to uninjured blood vessels is possible using a replication deficient adenoviral vector (Mazur et al. 1994). Using a respiratory syncitial virus promoter, peak gene expression occurred on day 7 and was no longer evident by day 28 (Lemarchand et al. 1993). Similar results were found in balloon injured rat carotid arteries (Landau et al. 1995; Lee et al. 1993).

Gene Transfer of Skeletal Troponin C to Rabbit Myocardium.

Data from transgenic mouse studies and protein substitution studies suggest that sTnC may confer advantageous properties to myocardial tissue. These include increased calcium sensitivity and relatively preserved contractile function in conditions of acidosis. These properties would be particularly relevant in the context of ischaemic myocardial dysfunction. It remains to be seen if skeletal troponin C confers an advantage in whole heart physiology in normal and pathological conditions. In particular, its potential to cause

abnormal relaxation has not been investigated. It is proposed to develop gene transfer methods which will cause skeletal troponin C expression in rabbit myocardium and to test the physiological consequences in isolated tissues.

Chapter 2

Methods

Adenoviral Vector Production

Materials

Unless otherwise stated, tissue culture media was obtained from Gibco BRL (Life Technologies Ltd, Paisley), tissue culture grade chemicals from Sigma (Sigma-Aldrich Co Ltd, Irvine) and tissue culture plasticware from Corning (Corning Incorporated, Corning, USA). Dishes 140mm x 20mm for large-scale viral propagation were obtained from Nunc (Nalge Nunc International, Rochester, NY, USA). All solutions used were prepared using microbiology grade chemicals and distilled water. After preparation they were sterilised by autoclaving or filtration through a 0.22μM filter. Nobel Agar 3.2% and Tris Saline (137 mM NaCl, 2.7mM KCl, 28mM Tris Base; pH 7.4) were stock items produced in the Department of Virology, University of Glasgow.

Frozen 239 Cell Stock

Human embryo kidney 293 cells were obtained at passage 28 (Microbix, Toronto, Canada). The passage number indicates the number of times the cells have undergone subculture since they were originally transformed by sheared Adenovirus type 5 DNA (Graham et al. 1977). A 2ml vial of cells was taken from storage in liquid nitrogen and rapidly thawed in a water bath at

37°C. The surface of the vial was then disinfected with 70% ethanol and all further procedures carried out within a Class II microbiological safety cabinet. Growth Medium (Minimum Essential Medium with Earle's Salts and Glutamine, 10% by volume Foetal Bovine Serum and Penicillin 90 iu/ml, Streptomycin 90μg/ml) was used to resuspend the cells. The cells were transferred to a sterile tissue culture flask with a surface area of 75-cm^2 and Growth Medium added to make a final volume of 20ml. After 6 hours of incubation (at 37°C, 5%CO_2) the cells became attached to the flask. The Growth Medium was changed to remove the cryopreservative (Dimethyl Sulphoxide). After 2-4 days a monolayer of cells typically covered 70-90% of the surface area of the tissue culture flask.

Subculture of 293 Cells

The cells were maintained in a subconfluent logarithmic growth phase by subculturing every 3-4 days. The cells were detached from the tissue culture vessels by washing twice with Citric Saline (135mM KCl, 15mM Tri-sodium Citrate) and incubating at 37°C for 5 minutes or until the cells freely detached from the surface of the culture vessel. The cells were then dispersed in fresh Growth Medium before distribution to three new culture vessels. Cells were not used beyond passage 50.

Maintaining Frozen Stocks

At passage 31 a proportion of the cells was refrozen for future use. These cells were detached using Citric Saline as above. They were resuspeded in

Foetal Bovine Serum with 10% Dimethyl Sulphoxide (DMSO). The vials were then frozen in a Cryol Freezing Container (Nalgene Nunc, Rochester, NY, USA) at –70°C for 48 hrs. A single vial was thawed and checked for cell viability prior to transfer of the remaining vials to liquid nitrogen for long-term storage.

Small-scale Propagation of Adenoviral Vectors

Small-scale propagation of adenoviral vectors was undertaken using 60mm diameter tissue culture dishes or tissue culture flasks with a surface area of 175cm^2. A tissue culture flasks with a surface area of 175cm^2 can grow a maximum of $7x10^7$ cells. However, this capacity is only possible if the cells are grown as a confluent monolayer. When the monolayer of 293 cells reached 70% confluency, the Growth Medium was aspirated and 1ml of Tris Saline containing stock adenoviral vector at a multiplicity of infection of 3 plaque forming units (pfu) per cell was carefully added.

To promote efficient transfection, cells were then incubated at 37°C 5%CO_2 for 45 minutes before adding Growth Medium. After 1-3 days, cells infected with viral vector exhibited a cytopathic effect, characterised by obvious rounding and eventual detachment from the culture vessel. The cells were harvested when at least half their number showed evidence of a cytopathic effect and before significant numbers spontaneously lysed and lost their viral load to the Growth Medium. At this point a cell scraper was used to manually detach all cells from the tissue culture vessel. The resuspended cells and growth media were transferred into sterile 50ml conical polypropylene tubes

and centrifuged at 1000 rpm for 10 minutes (Sigma Laborzentrifugen 4K15). The supernatant Growth Medium was discarded and the remaining cell pellet resuspended in 1 ml of Tris Saline and transferred to 1.5ml Eppendorf tubes. The virus within the 293 cells was then released into the Tris Saline by freeze-thaw cell lysis. Each Eppendorf tube underwent 3 cycles of freezing on dry ice and thawing in a 37°C water bath, vortexing vigorously between cycles. The Eppendorf tube was then centrifuged at 6000 rpm for 5 minutes (Biofuge 15, Heraeus Instruments, Hanau, Germany) to clarify the supernatant. The resulting crude lysate supernatant was then stored at –20°C or immediately further purified using a caesium chloride gradient (see below).

Large-scale Propagation of Adenoviral Vector

Large-scale propagation of adenoviral vector was similar to small-scale propagation. However, 14cm diameter dishes were used in place of flasks. Seventy such dishes occupy a similar volume to 24 175cm^2 flasks and gave 2.5 times more surface area for cell growth.

Purification by Caesium Gradient

Viral vector was isolated from crude lysate by ultracentrifugation on a 1.3 to 1.4 g/ml CsCl step density gradient at 100,000 g for 90 min at 4°C. Ultraclear centrifuge tubes (Sorval Instruments, Du Pont) were loaded with 2mls of CsCl (0.05M Tris containing 4.25M CsCl; pH 7.9). 3mls of CsCl (0.05M Tris containing 2.8M CsCl; pH 7.9) was carefully layered on top, followed by 2ml of Glycerol (0.1mM EDTA, Glycerol 40% w/v, 0.01M Tris; pH7.9) and then finally

7 ml of crude lysate supernatant and the tube weights balanced with Tris Saline before transfer to a TST41 rotor for ultracentrifugation at 25000 rpm for 90 minutes at 4°C. Following this, a distinct band, containing viral vector, was visible in the lowest most layer of the tube with cellular debris apparent above. The virus was isolated by puncturing the tube below the band with a 19-gauge hypodermic needle and drawing the virus into a 2ml syringe together with the surrounding caesium chloride solution. The virus was desalted by dialysis in a Collodion bag (Sartorius AG, Göttingen, Germany) against a total of 5L of dialysate buffer (0.01M Tris, 0.001M EDTA, pH 7.9, 4°C) overnight. The desalted virus was then transferred into Eppendorf tubes and stored at -20°C.

Quality Assurance

Background

For each of the adenoviral vectors used, a *Hin*DIII restriction enzyme DNA fragment pattern was known for the stock vector. Prior to use, all virus produced was subjected to restriction enzyme DNA analysis and the pattern produced compared with that from the stock vector and with that from replication competent wild type virus.

DNA Isolation

To 340μl of crude lysate supernatant was added, 20μl 100mM EDTA, 20μl 10% SDS, 20μl Proteinase K (20mg/ml), giving a total volume of 400μl in a 1.5ml Eppendorf tube. The mixture was incubated at 37°C for at least 4hrs.

Proteinase K was used to disrupt the viral capsid and the proteins bound to the viral DNA. Contaminating proteins and high molecular weight DNA was removed by two cycles of phenol extraction. Liquefied Phenol washed in Tris Buffer (Fisher Scientific, Loughborough) was added to an equal volume of Proteinase K digested lysate. After mixing, the organic and aqueous phases were separated by centrifugation at 6000rpm for 10 min (Biofuge 15, Heraeus Instruments, Hanau, Germany). The final aqueous phase was drawn off and mixed in a fresh Eppendorf tube with an equal volume of chloroform:Iso-amyl-alcohol (24:1). Again, the organic and aqueous phases were separated by centrifugation and the aqueous phase containing the viral DNA carefully drawn off.

Ethanol Precipitation

The aqueous phase containing viral DNA was mixed with 2.5 times by volume of absolute alcohol and 0.1 times of 3M Sodium Acetate pH5.4 by volume. The DNA was precipitated by cooling to –70°C overnight and pelleted by centrifugation at 15000rpm 4°C, for 10 minutes (Biofuge 15, Heraeus Instruments, Hanau, Germany). The liquid phase was discarded and the DNA pellet allowed to dry for 20 minutes at room temperature before resuspension in an appropriate volume of sterile water. For caesium purified virus the method of DNA extraction was the same except that only 100μl of virus was used and the volume made up to 340μl by addition of 240μl of Tris Saline.

*Hin*DIII Restriction Enzyme Digest

The DNA pellet was dissolved in 33μl of distilled water to which was added 1μl of RNAase, 4μl of 10X React 2 buffer (GibcoBRL) and 2μl of *Hin*DIII restriction enzyme (GibcoBRL) giving a total volume of 40μl. This was incubated at 37°C for at least 4 hrs. 10μl of loading buffer was added (0.25% Bromophenol Blue, 40% w/v glycerol) and the mixture loaded into an agarose gel for electrophoresis.

Agarose Gel Electrophoresis

The fragmented DNA product of the restriction enzyme digest was loaded into a well on a 0.8% (w/v) agarose gel made with 0.5X TBE (44.5mM Tris Base, 44.5mM Boric Acid 2mM EDTA pH8) and Ethidium Bromide 1μg/ml. Electrophoresis at 25-100V was undertaken until sufficient resolution of DNA bands was obtained, typically 75V for 4 hrs. A 1Kb DNA ladder (Gibco BRL) was run concurrently.

Taq DNA Polymerase Amplification of Adenoviral Vector DNA Fragments.

PCR was used to detect and amplify two different DNA fragments. The first was a fragment from the beta-galactosidase gene. The second was a fragment from the E1 region of the wild type adenovirus. The primers used were as follows:

Adenovirus Serotype 5 E1 region		Estimated Melting Point
Ad5-E1a629Forward	5'ATCGAAGAGGTACTGGCTGA	60°C
Ad5-E1a1045Reverse	5' CCTCCGGTGATAATGACAAG	60°C

Beta-galactosidase (LacZ)		Estimated Melting Point
LacZ 645 Forward	5'GCATAAACCGACTACACA	52°C
LacZ 1218 Reverse	5' GCTTCATCCACCACATAC	54°C

Oligonucleotide primers were obtained from Sigma-Genosys Ltd, Cambridge.

Each PCR reaction was undertaken in 500μl thin walled PCR tubes. Each tube contained 5μl 10X PCR Buffer (100mM Tris-HCl, pH8.3, 500mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin) 8μl of dNTP (10mM dATP, 10mM dCTP, 10mM dGTP, 10mM TTP) 1μM of each Primer, 0.5μl of *taq* DNA Polymerase (2.5units) (Sigma Aldrich, Poole, Dorset) and one tenth of the DNA from a small-scale viral propagation. Distilled water was added to a final volume of 50μl. The reagents were mixed on ice, adding the *taq* polymerase as the final step. The tubes were immediately transferred to a top heated Hybaid Omn-E thermocycler, pre-heated to 95°C. For beta-galactosidase fragment amplification the following program was used: Denaturing temperature of 95°C for 4 min then 30 cycles of 92°C for 1 min, annealing at 56°C for 1min and extension at 72°C for 1 min. The annealing temperature was above the estimated melting point for the primers used but this programme had been successfully employed within the group by Yvonne Alexander when working with genomic mouse DNA. Using the E1 specific primers the following program was used: Denaturing temperature of 95°C for 4min then 30 cycles of 94°C for 30 sec, annealing at 62°C for 30 sec and extension at 72°C for 1 min. The amplified DNA was evaluated by agarose gel electrophoresis.

Calculating Viral Concentration by Plaque Assay

The concentration of virus produced was determined by plaque assay. This was performed in duplicate and the result expressed as the mean of the two values. For each assay 293 Cells were grown to 80%-90% confluency in 60mm tissue culture dishes. A vial of viral vector was thawed and 10μl of virus was serially diluted by a factor of 10 using Tris Saline over the range 10^{-2} – 10^{-14}. A fresh pipette tip was used at each dilution. Growth Medium was removed from the tissue culture dishes and 100μl of a viral dilution carefully dripped onto the cells of an appropriately labelled dish. The cells were then incubated at 37°C 5% CO_2 for 45 minutes before being overlaid with 2mls of Agar Mixture (38ml Growth Medium, 2ml Foetal Bovine Serum, and 10ml molten 3.2% Noble Agar). After three days of incubation, a further layer of Agar Mixture was added. A viral plaque was characterised by a new hole in the cell sheet with cells at the margin showing features of a cytopathic effect. The number of viral plaques was counted 7 days following infection and the concentration of viral vector expressed in plaque forming units per ml of undiluted virus (pfu/ml).

Plaque Purification

The stock viral vectors were derived from single plaques picked from agar overlay dishes produced as described above. Only dishes with fewer than 10 plaques were used. The dishes were allowed to incubate for up to 9 days. The

positions of the plaques were marked and the dishes allowed to cool at 4°C for 10 minutes. A sterile Pasteur pipette with a bulb was used to aspirate both the cells and surrounding agar of a single plaque. The material was then resuspended in Tris Saline and agitated before being used to infect a 175-cm^2 flask of 293 cells. For vectors encoding beta-galactosidase, positively staining plaques were identified by X-gal staining. 250mg of X-gal was dissolved in 5ml of tissue culture grade DMSO. Each 60mm dish had 100μl of X-gal solution carefully dripped onto the surface of the agar. The plates were incubated for 4 hours and the number of positively staining areas counted. Where the number of positively staining areas numbered fewer than ten, plaques were individually aspirated and stored for future use.

Adenoviral Vectors

Introduction

Six adenoviral vectors were available and are described below. All were E1a deleted, replication deficient and serotype 5.

Adenoviral Vector Expressing Beta-galactosidase

CMV-LacZ Ad5

This vector encoded beta-galactosidase under the control of the cytomegalovirus promoter. This was a kind gift from Ron Hay, St Andrews University, UK and had been constructed using the plasmids available from

Microbix (Microbix Inc, Toronto, Canada). The Ad5 genome plasmid pJM17 has a 4.4Kb insert from a bacterial plasmid inserted at the *Xba*I site in the E1 region of the wild type adenovirus genome Ad5-dl309. The insert allows replication of the plasmid pJM17 within bacterial culture systems. However, pJM17 exceeds the packaging capacity of the adenovirus capsid. When pJM17 is co-transfected into HEK293 cells with the appropriate shuttle plasmid encoding beta-galactosidase, replication deficient adenoviral vector can result. On the shuttle plasmid, the gene of interest is flanked by DNA sequences that are homologous to sequences within pJM17. By a process of homologous recombination the bacterial DNA insert and E1a region is replaced by the smaller sequence, including the gene of interest, from the shuttle plasmid. The resulting plasmid is smaller and can be packaged within the adenoviral capsid. Although E1a deleted and replication defective, the recombinant virus can be propagated in the complementing, E1a expressing HEK293 cell line.

RSV-nLacZ Ad5

This vector encoded a nuclear localising beta-galactosidase under the control of the Rous Sarcoma Virus promoter. This was a kind gift from Roger Hajjar, Boston, USA. This vector had been constructed to be both E1a and E3 deficient (Dong et al. 1996). The method used by Dong is briefly described. The following plasmids were obtained: pSP72 (Promega, Madison, WI); pAdRSV4 (Dr. Blake Roessler, University of Michigan); pLZ11 (Dr. Joshua Snes, Washington University). A *Bgl*II fragment containing the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter and SV40 polyadenylation sequence (termed "SvpA") was excised from pAdRSV4 and ligated into *Bam*HI-digested pSP72. The resulting plasmid, designated pSP72BglII, was

digested with *Sma*I and *Bam*HI then ligated to a blunt-ended, *Bgl*II-*Xba*I fragment of pLZ11. This *Bgl*II-*Xba*I fragment of pLZ11 contained a nuclear-targeted Escherichia coli beta-galactosidase gene (nLacZ). The plasmid product of the ligation of the *Bgl*II-*Xba*I fragment to pSP72BglII, termed pSP72β-Gal, contained nLacZ fused to SvpA. The plasmid pAdRSVnLacZ was constructed as follows. pSP72β-Gal was digested with *Bgl*II and *Bam*HI, releasing the nLacZ sequences. This fragment was ligated into the *Bam*HI site of the pAdRSV4, creating an expression cassette consisting of the RSV LTR promoter driving nLacZ-SvpA. The adenoviral vector was generated by cotransfection of linearised plasmid pAdRSVnLacZ with the 35-Kb *Cla*I of Ad-dl327 (an E3-deleted Adenovirus) in 293 cells. Freeze-thaw lysates made from plaques were tested for their ability to transfer beta-galactosidase expression to 293 cells. This adenoviral vector was successfully used by Dong et al to infect calf pulmonary artery cells *in vitro* and rat coronary arteries *in vivo.*

Adenoviral Vectors Encoding Skeletal Troponin C

Obeid Khan and Yvonne Alexander constructed all the original vectors encoding skeletal troponin C under the supervision of Howard Prentice (Khan 1999). The plasmids encoding skeletal Troponin C and the Human Cardiac Actin Promoter (pLK419 and pLK350 respectively) were obtained from Larry Kedes, University of Southern California, CA, USA. The sTnC cDNA was subcloned into the vector pLECM using PCR amplification and cutting both the PCR product and pLECM with the restriction enzymes *Eco*RI and *Bgl*II. The resulting plasmid was designated pLECM/sTnC. To obtain a sTnC clone

with SV40-polyA sequence as the 3' end of the gene, a PCR product of pLECM/sTnC was inserted into pCA13 using the restriction enzymes *Eco*RI and *Bam*HI. This plasmid was designated pCA13/sTnC. The restriction enzymes *Bst*EII and *Avr*II were used to isolate the cDNA for sTnC which was then inserted into the vector pSP72 following digestion of this vector by *Sma*I. The resulting plasmid was designated pSP72/sTnC. For the vectors encoding the HA tag, a 12 codon sequence was added to a truncated PCR product of the sTnC. Sequencing of the resulting plasmids was used to ensure appropriate orientation of inserted fragments. Adenoviral vectors were produced by calcium phosphate transfection of HEK293 cells with both the appropriate shuttle plasmid and the large adenoviral plasmid pJM17 (Microbix Biosystems Inc, Toronto, Canada). Resulting plaques were screened by restriction enzyme analysis and PCR using E1 region specific primers.

CMV-sTnC Ad5

This vector encoded human skeletal troponin C under the control of the cytomegalovirus promoter. The cDNA had been isolated from a human muscle DNA library (Gahlmann et al. 1988). The virus was constructed using a plasmid pLK419 (Larry Keddes, University of South California, USA)

CMV-sTnC-Δ9-HA Ad5

This vector was similar to CMV-sTnC Ad5. However, the sequence encoding the C-terminal nine amino acids had been removed and replaced with a 12 amino-acid sequence. This new sequence encoded 3 linker amino-acids attached to a 9 amino-acid haemagglutinin antigen motif.

HCA- sTnC Ad 5 and HCA- sTnC-Δ9-HA Ad5

These vectors were similar to their CMV promoter equivalents but gene expression was under the control of the human cardiac actin promoter.

CMV-sTnC-Δ9-HA Ad5 and HCA-sTnC-Δ9-HA Ad5 were used by Dr Khan to infect NIH3T3 mouse fibroblasts cells in culture. A western blot for the presence of HA tag was positive in fibroblast cells infected with CMV-sTnC-Δ9-HA Ad5 but negative for cells infected with HCA-sTnC-Δ9-HA Ad5 and in uninfected cells. Both vectors were used to infect rat neonatal cardiocytes. Immunostaining for the HA product was positive in CMV-sTnC-Δ9-HA Ad5 infected cardiocytes and to a lesser extent HCA-sTnC-Δ9-HA Ad5 infected cardiocytes, but was negative in negative control groups. Dr Khan concluded that the vectors could cause foreign gene expression and that the HCA-sTnC-Δ9-HA Ad5 vector demonstrated characteristics of tissue specific expression. However, from these experiments it was not known if the HA tagged product integrated into the sarcomere of infected myocytes. The C-terminal 9 amino-acids had been replaced by a 12 amino-acid sequence including the 9 amino-acid sequence for the HA tag. The C-terminal of TnC sits close to and interacts with the other troponin sub-units and the possibility remained that the lengthened HA tagged mutant may not integrate into the sarcomere. In retrospect, confirmation of integration of sTnC-HA into the sarcomere in cell culture should have been sought prior to proceeding to *in vivo* functional experiments.

Delivery Techniques

Surgical Methods

Male adult New Zealand white rabbits supplied by Harlan-Olac Ltd weighed 2.5-3.5Kg at the time of surgery unless otherwise stated. All animal procedures were in accordance with Home Office requirements and all surgery was undertaken under general anaesthesia.

Induction of General Anaesthesia

Surgical procedures were performed under the supervision of Dr Martin Hicks, Glasgow Royal Infirmary. Fifteen minutes prior to any surgical procedure, animals were premedicated with 0.4ml/kg of intramuscular Hypnorm (Fentanyl Citrate 0.315 mg/ml, Fluanisone 10mg/ml, Janssen Animal Health, Beerse, Belgium). Following adequate sedation with Hypnorm, the surgical area was shaved and a solution of 0.5% chlorhexadine in 70% alcohol applied. A peripheral venous cannula was secured by introducing a 23-gauge butterfly needle into the auricular vein. For several minutes prior to endotracheal intubation the sedated animals breathed a mixture of oxygen and nitrous oxide by facemask. (Oxygen 1L/min, NO 0.8L/min mixed by a Boyle's anaesthetic machine). Immediately prior to intubation the animal was deeply sedated using Hypnovel (Midazolam 10mg/2ml, Roche, Welwyn Garden City) 0.17mg/kg given as an intravenous bolus. A paediatric laryngoscope was used to visualise the epiglottis and vocal cords. Xylocaine throat spray (Astra Pharmaceuticals, Langley) was used as a local anaesthetic to the vocal cords

before a paediatric endotracheal tube (3.0-3.5mm Portex, Berk Sur Mer, France) was introduced and the trachea intubated under direct vision. Ventilation of both lungs was confirmed by auscultation. Excessive advancement of the tube can result in selective ventilation of only one lung. The animal was mechanically ventilated at a minute ventilation of 1.2-1.6L using oxygen and nitrous oxide mixture as above. Maintenance of general anaesthesia was achieved using 1%-2% Halothane (Zeneca Ltd, Macclesfield).

Central Vessel Cannulation

Following a small right paratracheal incision and longitudinal dissection of the paratracheal musculature, the internal jugular vein was identified. The vessel was isolated by means of a silk tie proximally (Mersilk) and a bulldog clip distally. The vessel was then entered and a cannula secured with silk suture prior to removal of the bulldog clip, after which the cannula could be advanced if required. Cannulation of the common carotid artery was similarly achieved. The common carotid artery was carefully isolated to avoid damage to the adjacent vein and vagus sympathetic trunk.

Left Lateral Thoracotomy

The skin was incised along the line of the left 4^{th} intercostal space. The intercostal muscles were identified following dissection of the subcutaneous fat, fascia and serratus anterior muscle. The intercostal muscles were divided in two layers to facilitate an airtight seal upon subsequent closure. The pleural

cavity was entered by blunt dissection, minimising the risk of damage to the lung that could result in a postoperative pneumothorax. The 4th and 5th ribs were separated using a self-retaining rib retractor, giving adequate access for instrumentation within the chest. The left lung was manually collapsed and held in position with a small cotton swab. The animal was ventilated with 0.5-1cm H_2O of positive end expiratory pressure. The pericardium was opened with scissors at the level of the atrio-ventricular groove and the incision extended towards the apex as required, allowing the apex to be displaced from the pericardial sac. The position of the left ventricular apex was controlled using a transmural 4/0 Ethibond suture (Ethicon, NJ, USA). This gave adequate control for direct needle injection of adenoviral vector into the myocardium both at the apex and left baso-lateral wall. Following the required procedure, the apical tie was removed and haemostasis achieved by direct pressure. The heart was placed back into the pericardial sac. The swab holding the collapsed lung in place was removed and at least 3 interrupted 3/0 chromic catgut sutures used to appose the fourth and fifth ribs. The left lung was re-inflated before securing the chromic ties. The wound was then closed in layers using interrupted 3/0 Ethibond suture and the skin closed with a continuous subcuticular Dexon 2-0 suture (Sherwood Davis and Geck, St Louis, MO, USA).

Direct Needle Injection

At open thoracotomy, adenoviral vector was injected directly into the myocardium of the left ventricular free wall using a 26-gauge needle attached to a 100μl glass Hamilton syringe. The position of the heart was controlled

using an apical tie and the tip of the needle was bent to a right angle and introduced into the myocardium at an oblique angle. These precautions were taken in order to reduce mechanical trauma and to minimise the risk of injecting the vector into the ventricular cavity. The injection was deemed satisfactory if there was blanching of the surrounding myocardium at the time of injection. Before closing the chest, the position of the injection site was noted relative to the branching epicardial vessels.

Coronary Infusion Using a Swan-Ganz Catheter

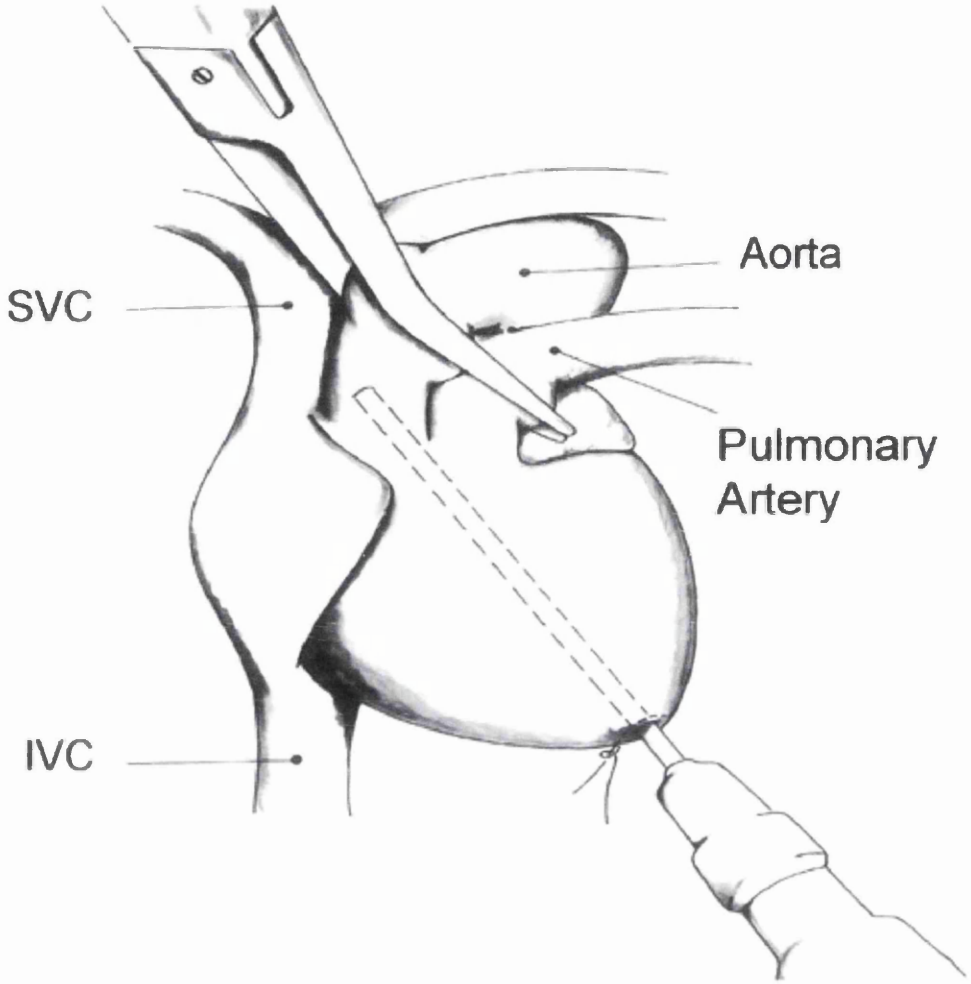
A Swan-Ganz catheter (sized 5-6 French) was introduced into the right common carotid artery via a right paratracheal skin incision. Under fluoroscopic guidance (Siemens GRA 1030, Siemens Elema, Sweden) the catheter was manipulated into the ascending aorta. The distal port was attached to a pressure transducer (Elcomatic EM 751A, Elcomatic Ltd, Glasgow) and pressure at the distal tip monitored on an oscilloscope trace (144 Monitor, Roche, Basel, Switzerland). A hard copy of the pressure tracing could be made using a paper recorder (Mingograf 803, Siemens Elema, Sweden). The Swan-Ganz catheter balloon was then inflated and occlusion of the ascending aorta confirmed by an increase in the aortic pressure tracing from the distal port and visually by injection of radio-opaque contrast below the balloon (Omnipaque, Nycomed, Birmingham). Having confirmed that the ascending aorta was effectively occluded, virus was then infused into the distal port and the catheter flushed with 0.9% saline. With an occluded ascending aorta above and a competent aortic valve below the virus was

injected into a space where the only means of escape was through the coronary arteries.

Introduction of Adenoviral Vector by Pulmonary Artery and Aortic Cross-Clamp

This method required an open thoracotomy and a left ventricular puncture. The method was first described in the rat and was adapted for use in rabbits where the heart and great vessels were exposed following a left 3rd rib-space thoracotomy (Hajjar et al. 1998). An inverted T skin incision was necessary for adequate access. The pericardial sac was opened and the left ventricular apex controlled using a silk suture. An 18-gauge cannula was introduced into the left ventricular cavity by direct puncture through the left ventricular apical notch. After removing the needle, the tip of the catheter was manipulated into the ascending aorta. The aorta and pulmonary arteries were simultaneously clamped using a large pair of forceps and the cannula withdrawn to lie immediately below the clamp but above the aortic valve (Figure 2-1). To avoid a prolonged period of ischaemia, the clamp was then briefly released before being tightly applied. Adenoviral vector was then injected as a bolus through the cannula and the clamp kept in place for 10 seconds after the injection. As an indicator of the effectiveness of the pulmonary clamp, it was noted if it was possible to draw blood back into the syringe following injection. After the clamp was released, the cannula was removed and haemostasis achieved by direct pressure. The chest was closed as previously described.

Figure 2.1. Method of viral introduction adapted from Hajjar (Circ Res 2000 Vol 86(6) p616-621) SVC – Superior Vena Cava, IVC – Inferior Vena Cava



Selective Coronary Cannulation

Selective cannulation of the coronary arteries proved possible using a custom made 3 French nylon coronary catheter with a hockey-stick tip (Royal Flush II Radiopaque Nylon Catheter, Type N3.0-21-20-P-NS-MAC1, William Cook Europe A/S, Bjaeverskov, Denmark, a kind gift from Professor Michael Marber, Rayne Institute, St. Thomas' Hospital, London). The catheter was introduced into the ascending aorta via the internal carotid artery. Heparinised

saline was then injected (1ml with 200iu mucous heparin Leo Labs Ltd, Risborough) before the catheter was manipulated into one of the coronary ostia under fluoroscopic guidance. The position of the catheter was confirmed by a small injection of radio-opaque contrast (<0.5ml). If necessary the catheter was advanced until it occluded the coronary artery. In this position, injected contrast material would remain in the coronary artery until flushed away with injected saline solution. Following injection of adenoviral vector the catheter was flushed with 1ml of heparinised saline and a small injection of contrast used to confirm the catheter position. The catheter was then removed and coronary patency confirmed by continuous screening until the contrast material flushed away.

Post-operative Analgesia

Immediately following surgery the animals received intramuscular Buprenorphine (0.15mg in 0.5ml as hydrochloride, Animalcare Ltd, York). This was repeated 16 hours later.

Histological Staining

Staining of Myocardial Tissue Blocks for Beta-galactosidase (Method 1)

Blocks of myocardium were immersed in Fixing Solution (Phosphate Buffered Saline; pH7.4 containing 2% formaldehyde and 0.2% gluteraldehyde) for 15 minutes. The Fixing Solution was removed and the cells washed 3 times with PBS. The tissue was then immersed in X-gal Stain (5mM Potassium Ferrocyanide, 3mM Potassium Ferricyanide, containing 1mg/ml X-gal dissolved in Dimethyl Formamide 0.1% v/v) and the dishes incubated at 37°C for 4-16 hours.

Staining of Myocardial Tissue Blocks for Beta-galactosidase (Method 2)

Myocardial tissue of 3mm thickness was stained as a block before histological sectioning. At room temperature, the tissue was rinsed in 0.1M Phosphate Buffer pH 7.3 (0.1M Sodium Phosphate Monobasic:0.1M Sodium Phosphate Dibasic, ratio 1:3 v/v) and then fixed for 30 minutes (0.1M Sodium Phosphate; pH7.3, 0.2% Gluteraldehyde, 5mM EGTA; pH 7.3, 2mM Magnesium Chloride). The tissue was then washed 3 times at 15-minute intervals in Wash Buffer (0.1M Sodium Phosphate; pH 7.3, containing 2mM Magnesium Chloride, 0.1% v/v deoxycholate and 0.2% v/v Nonidet-P40). The tissue was then stained with X-gal Solution (0.1M Sodium Phosphate; pH 7.3, containing 1mg/ml X-gal dissolved in Dimethyl Formamide, 5mM Potassium

Ferrocyanide, 3mM Potassium Ferricyanide, 0.1% v/v Deoxycholate and 0.2% v/v Nonidet-P40) for 16 hours at 37°C. After staining the tissue was kept in Wash Buffer at 4°C overnight before being taken for histological sectioning.

Staining by Coronary Infusion

To ensure that the full thickness of the myocardium was exposed to the staining solutions for beta-galactosidase expression, a coronary infusion method was developed. Hearts were removed under terminal anaesthesia. Each heart was suspended above a glass funnel by sliding the ascending aorta over a custom-made aluminium cannula and securing it with a Mersilk tie. Solutions were perfused under constant flow into the ascending aorta and down the coronary arteries at a rate of 20ml/min. The glass funnel below collected the effluent from the coronary sinus. The apex of the left ventricle was vented by piercing with a pair of watchmaker forceps. At room temperature the heart was perfused with 0.1M Phosphate Buffer pH 7.3 (0.1M Sodium Phosphate Monobasic:0.1M Sodium Phosphate Dibasic, ratio 1:3 v/v) for 10 minutes, followed by Fixing Solution (0.1M Sodium Phosphate; pH7.3, 0.2% Gluteraldehyde, 5mM EGTA; pH 7.3, 2mM Magnesium Chloride) for 12 minutes, and finally with Wash Buffer (0.1M Sodium Phosphate; pH 7.3, containing 2mM Magnesium Chloride, 0.1% Deoxycholate and 0.2% v/v Nonidet-P40) for 15 minutes. The heart was then stained by perfusion with X-gal Solution (0.1M Sodium Phosphate; pH 7.3, containing 1mg/ml X-gal dissolved in DMSO, 5mM Potassium Ferrocyanide, 3mM Potassium Ferricyanide, 0.1% v/v Deoxycholate and 0.2% v/v Nonidet-P40). The X-gal solution was heated in a water bath to 37°C and perfused at 20ml/min. The effluent collected by the funnel was returned to the reservoir and re-circulated.

Each heart was infused with X-gal solution for 1 hour. After staining the tissue was kept in Wash Buffer at 4°C overnight before being taken for histological sectioning.

Histological Sections

Niall Whyte of the Department of Pathology, Western Infirmary, Glasgow, performed all processing of tissue blocks for histological sectioning and cutting of histological sections. Standard 6-micron sections were cut and stained with Haematoxylin and Eosin (H&E). Unstained sections were left uncovered on aminopropyltriethoxysilane (APES) covered glass slides for future immunohistochemistry. When unfixed sections were required, frozen sections were cut using a cryotome.

Detection of HA-tag by Immunohistochemistry

Some tissues were exposed to vector encoding gene products which included the haemagglutinin antigen (HA) motif. The antibodies used for detection of the HA epitope were supplied by Boehringer Mannheim. A literature review and the technical assistance of Boehringer Mannheim failed to find an established method for using these antibodies on sections of rabbit myocardium, and therefore the following method was developed. Anti-HA high affinity Rat monoclonal antibody (Clone 3F10) was used as the primary antibody in a three-step procedure. Anti-rat Ig-biotin, F $(ab')_2$ fragment was used as a secondary antibody. Streptavidin biotinylated peroxidase was used

to conjugate to the secondary antibody. Finally, the sections were developed using 3',3'-diaminobenzadine (DAB).

Both frozen sections and wax sections were used. The wax sections were dewaxed by placing them in Xylene for 10 minutes, rinsing 3 times in alcohol and once in water. All slides were placed in 3% Hydrogen Peroxide for 10 minutes to inhibit endogenous peroxidase activity. The slides were rinsed with water and then TBS (0.05M Tris, 145mM NaCl; pH 7.6) containing 0.5% Tween 20. The antibodies were diluted with a solution of TBS containing 0.5% Tween 20 and 1% Bovine Serum Albumin (BSA). A wax pen was used to draw around the section on each slide. Non-specific binding of protein was blocked by covering each section with 100μl of 1% BSA for 15 minutes. This was dripped off and 100μl of primary antibody was placed on each slide at varying dilutions. The dilutions used were 1:50, 1:100 and 1:200. At each stage the slides were allowed to sit in a humidifying box for 30 minutes before being washed with TBS containing 0.5% Tween. A 1:100 dilution of Anti-rat Ig-biotin, F $(ab')_2$ fragment was used as a secondary antibody and a 1:100 dilution of ABC (Peroxidase complex by Vector Laboratories Inc, Burlingame, CA, U.S.A.) used for the third step. The slides were washed 3 times with TBS containing 0.5% Tween before being stained with 3',3'-diaminobenzadine (DAB). The DAB staining was performed in a fume cupboard using a solution of 0.05M Tris buffer pH 7.6 containing 0.33mg/ml DAB, 0.067mM imidazole and 0.6% hydrogen peroxide.

Tissue obtained from a rabbit heart that had been directly injected with CMV-sTnC-Δ9-HA Ad5 five days before sacrifice served as a positive control. Tissue obtained from rabbit myocardium, which had not been injected with viral vector, was used as a negative control. The primary antibody was

omitted from a full set of slides and these served to control for the possibility of the secondary antibody having non-specific binding to rabbit myocardium.

Quantitative Analysis of Histological Sections

Using light microscopy (Model DM1L, Leica, Wetzlar, Germany), H&E stained histological sections were examined for evidence of foreign gene expression, cell death and inflammatory infiltrate. The proportion of myocardium with detectable foreign gene expression was determined by point counting. An England Finder Grid (Graticules Pyser-SGI Ltd, Tonbridge) was inverted and randomly placed over the histological slide. At 40X magnification both the histological section and the grid were in focus. At each intersection of the grid, the underlying cell type was identified by its morphological features. Cardiomyocytes were identified by their cross striations(Plate 2.1; Plate 2.2). The number of cardiomyocytes staining positively for the foreign gene product was noted together with the total number of intersections counted. Where a cell did not overlay the intersection, the cell to the immediate upper left was counted. This was performed separately for the right ventricular free wall, trabeculae rich basal right ventricle and left ventricular free wall. For each region, the count was performed five times.

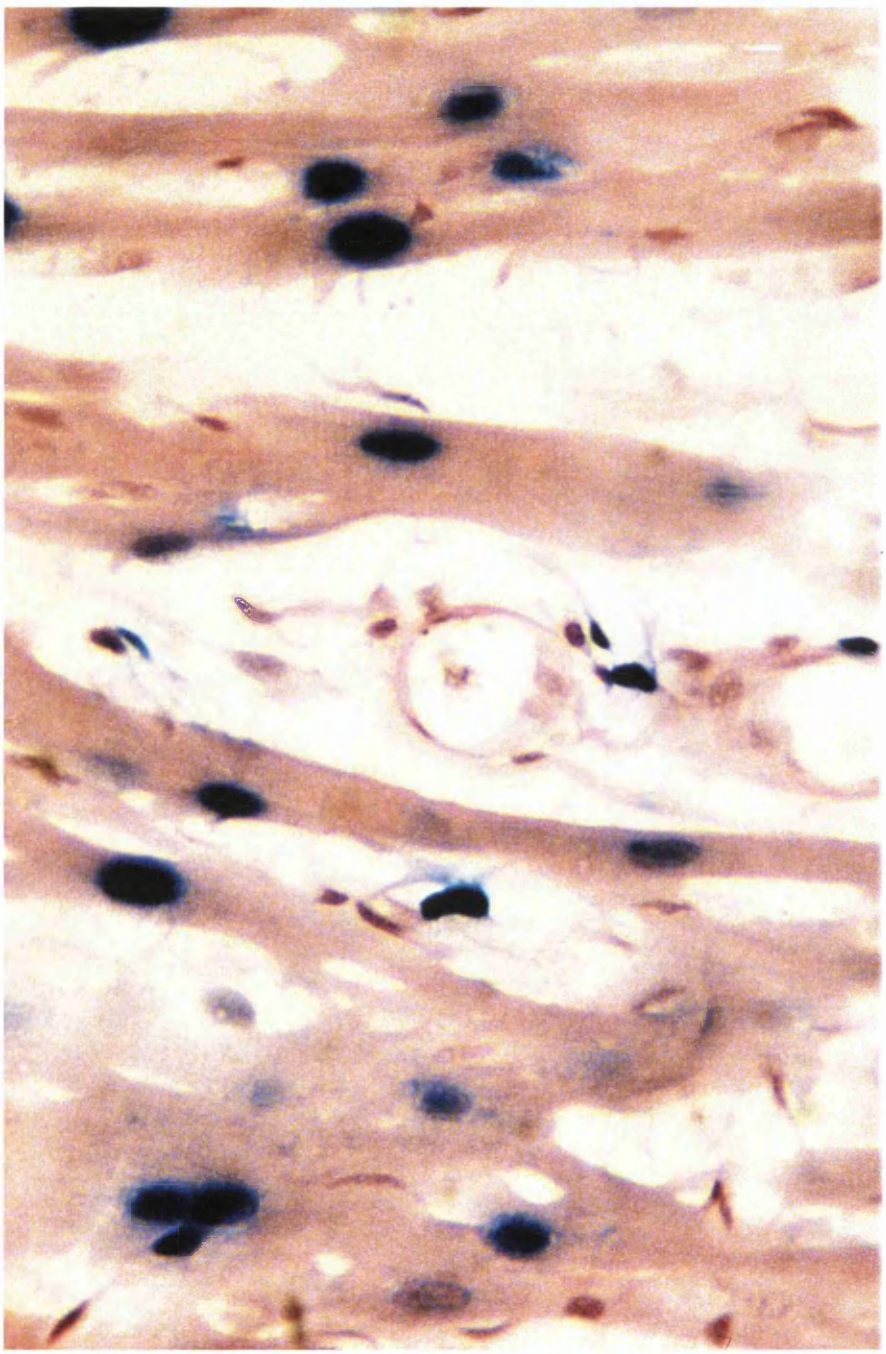


Plate 2.1. An example of a histological section (magnification X100) representative of positively staining (blue) nuclei in the myocardium of a rabbit 5 days following the introduction of vector encoding nuclear localising beta-galactosidase. Myocytes can be identified by their cross-striations

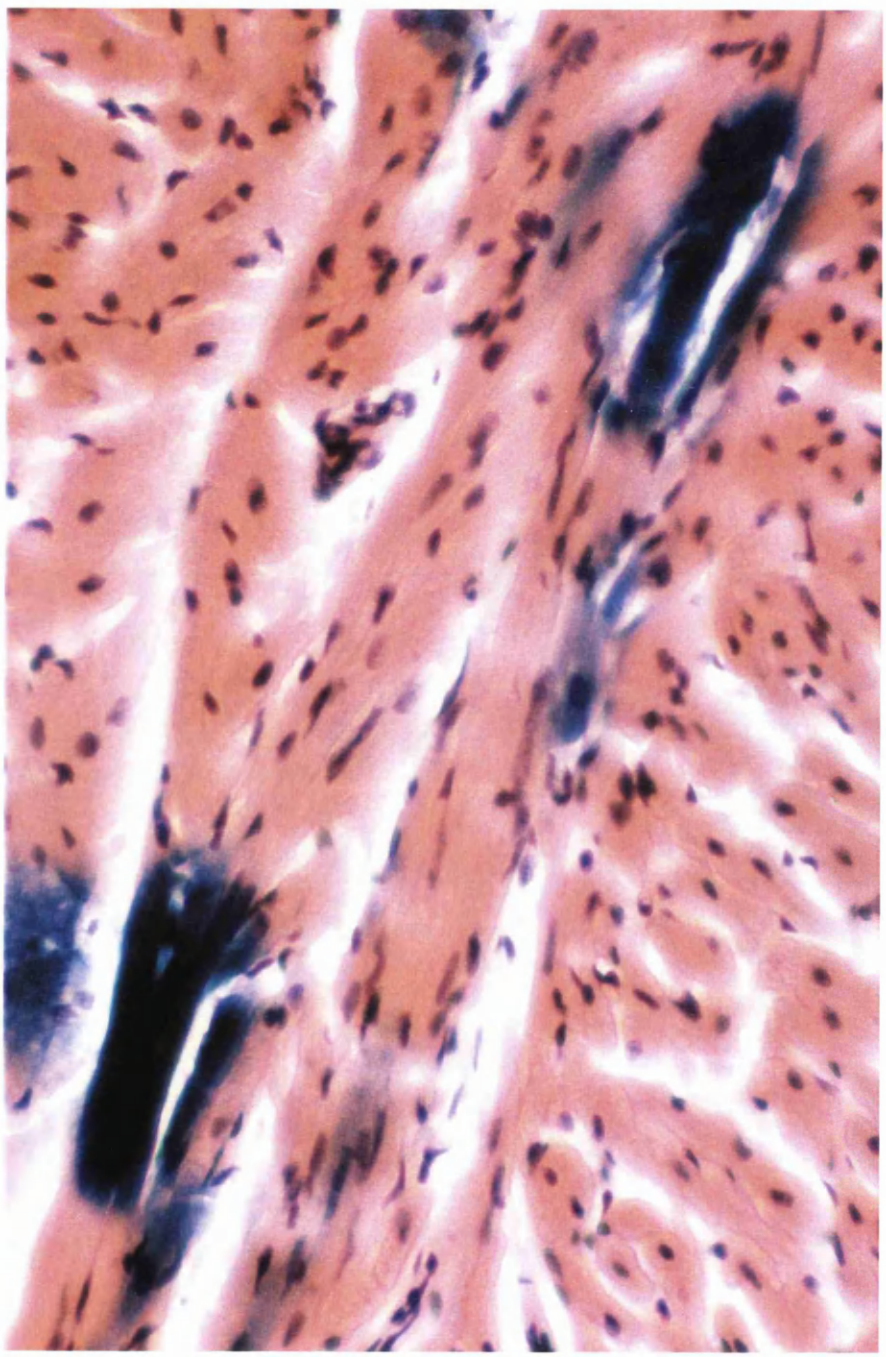


Plate 2.2. An example of a histological section (magnification X100) representative of positively staining (blue) cells in the myocardium of a rabbit 5 days following the introduction of vector encoding beta-galactosidase. Spillage of the X-gal stain product to neighbouring cells is seen.

Photography

A 35mm SLR camera (Model XR-X 3PF, Ricoh, Tokyo, Japan) was attached to the light microscope. Tissue culture cells and sections were photographed using Kodak ET75 (Ektachrome-tungsten) slide film.

Physiology Methods

Calcium Sensitivity of Trabeculae

Animals were sacrificed by intravenous Euthatal (0.5ml/kg) five days after viral introduction. The hearts were removed following a right parasternal incision and immediately placed in Modified Ringer's solution (150 mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES, 10mM Glucose; pH 7.4) containing 30mM 2,3-Butanedione Monoxime (BDM). The right ventricle was freed from the intra-ventricular septum anteriorly. Using a dissecting microscope, right ventricular trabeculae of suitable proportions were identified and carefully dissected. Only trabeculae with a diameter of 150μm or less were used for physiological experiments. Selected trabeculae were mounted between two nylon monofilament snares. One snare was fixed and the other attached to a force transducer (Akers AE875, SensorNor a.s. Norway). The whole assembly was mounted on a micromanipulator (Narashige MM3) which allowed movement in 3 planes. A second micromanipulator facilitated fine adjustments to sarcomere length.

Production of Skinned Fibres

Complete disruption of surface and intracellular membrane diffusion barriers was achieved by immersing the preparation in a well containing 10R solution (Table 2.1), 1% Triton X-100 for 40 minutes.

Measuring Calcium Sensitivity of Skinned Fibres

A modified Vickers M-16 microscope with differential interference contrast optics was used to examine each trabecula and set the sarcomere length to 2.2μ. Solution changes were achieved using a computer controlled step motor driving a block of Perspex into which had been cut a series of wells. Solution 10R contained the calcium chelator EGTA at a concentration of 10mM (Table 2.1.). When permeabilised trabeculae were exposed to this solution, the free calcium was low and the preparation remained relaxed. Solution 10A contained CaEGTA at a concentration of 10mM. By mixing solutions 10A and 10R, solutions of differing free ionic calcium were produced. The wells contained solutions of varying calcium concentrations prepared at pH 7.0 and pH 6.5. (Table 2.2). Preparations were moved to the well of the test calcium concentration after equilibration in solution 0.2R, thereby producing a rapid increase in the calcium buffer concentration at the same time as the free calcium concentration.

Table 2.1. Composition of solutions 10A, 10R and 0.2R in mM.

Solutions	*K+*	*Mg2+*	*ATP*	*CrP*	*Na+*	*CaEGTA*	*EGTA*	*Hepes*	*pCa pH 7.0*
10A	140	7	5	15	40	10	0	25	4.29
10R	140	7	5	15	40	0	10	25	9.63
0.2R	140	7	5	15	40	0	0.2	25	7.91

Table 2.2. Composition of solutions for bath change system (10A Full is 10A + 50µL 10mM $CaCl_2$

Ratio 10A:10R	*1:1*	*2:1*	*5:1*	*8:1*	*15:1*	*10A*	*10A Full*	*10R*	*0.2R*
pCa @ pH 7.0	6.32	6.02	5.62	5.42	5.15	4.29	4.06	9.63	7.91
pCa @ pH 6.5	5.36	5.05	4.67	4.47	4.24	3.79	3.71	8.66	6.96

The method employed for calculating the free calcium ion concentration has been reported elsewhere (Harrison, Lamont, & Miller 1988). REACT is a computer program which calculates the equilibrium position for solutions containing a mixture of several ions and polyvalent buffers of the type used in these experiments (Miller & Smith 1984; Smith & Miller 1985). All physiological experiments were performed at room temperature (20-22°C).

After permeabilisation, the preparation was transferred to a thin glass-sided chamber. A Vickers M-16 microscope with Differential Interference Contrast optics attached to a video camera was used to examine and set sarcomere length. A graticule was placed within the video tube and the fine micromanipulator adjusted to give a sarcomere length of 2.1-2.2μm. The total length and width of the preparation was measured using the graticule. The depth was determined by focusing backwards and forwards and reading the micrometer on the microscope stage. The isometric force produced by the preparation was recorded on an Apple Macintosh computer using a MacLab analogue to digital converter (ADI Instruments Pry Ltd, Australia) and Apple Macintosh Chart software. Off-line Computer analysis was performed. Force generation for each calcium concentration was expressed as a proportion of the maximal force generated for a saturating calcium solution. A computer programme was used to fit a Hill equation curve through the data obtained.

Hillfit Computer Macro

The Hill equation $C_x/C_{max} = [Ca_x]^h/(EC_{50}{}^h+[Ca_x]^h)$ describes the relationship between steady-state force and Ca^{2+} concentration where C_{max} is the force at a saturating Ca^{2+} concentration; EC_{50} is the Ca^{2+} concentration that gives 50% of Cmax and h is the Hill coefficient. A macro running in the Mac application 'Igor Pro' (Written by Dr D Miller, using a Levenberg-Marquadt least squares algorithm) was used to generate a best-fit curve to the Hill equation for each preparation examined. From the curve thus derived, the EC_{50} for each preparation could be calculated.

Statistical Methods

Statistical calculations and graphs were produced using SigmaStat software (SPSS, San Rafael, CA, USA). A Student two-tailed, unpaired t-test was used for continuous variables where the data were normally distributed. For non-parametric data, the Mann-Whitney test was applied. Rejection of the null hypothesis was accepted at $p<0.05$.

CHAPTER 3

Adenoviral Vector Production

Introduction

We wished to test the hypothesis that adenoviral mediated gene transfer of skeletal troponin C to myocardial tissue would result in resistance of the myocardium to the calcium desensitising effects of ischaemia in tissue expressing sTnC but not in controls. It has been reported that adenoviral vectors in themselves can alter the contractile characteristics of infected myocytes (Novotny, J., Gustafson, B., Kvapil, P., & Ransnas, L. A. 1994). It was for this reason that the control groups should have included a group infected with empty adenoviral vector. Beta-galactosidase is thought to be physiologically inert and is not known to alter the contractile characteristics of infected myocytes. However, this is not certain and although we used an adenoviral vector encoding beta-galactosidase, a better adenoviral vector control would have been empty virus. It is possible that the method employed to introduce the vector, or the vehicle in which the vector was delivered, could have had an effect on the myocardial tissue. For these reasons, tissue from animals exposed to vehicle alone should have been investigated. Whilst developing the selective coronary injection method of adenoviral mediated gene transfer, one group was pre-injected with a solution containing serotonin. The lack of data from a control group injected only with vehicle was a serious shortcoming and limited the conclusions which could be drawn from these data. For the purpose of physiological investigation, a prerequisite was to achieve gene transfer to a large proportion of rabbit myocardium *in vivo*. Following gene transfer, it was anticipated that an interval of several days would be required to allow gene expression and accumulation of the gene

product prior to physiological investigation. Ideally, the vector employed would be of high titre, capable of efficiently infecting non-dividing cardiac myocytes and would not in itself evoke a significant inflammatory response prior to physiological investigation. The vector would have an insert capacity sufficient to accept genes of interest and their controlling regions. For reasons of safety, an absolute requirement was that the vector be replication defective and incapable of amplification in humans or live animals.

Background

In vivo gene transfer of rabbit myocardium by a replication defective adenoviral vector had been reported previously (Barr et al. 1994). Wild type adenovirus serotype 5 is a minor respiratory pathogen. It is a capsulated double stranded linear DNA virus capable of infecting and replicating in a wide variety of mammalian cells. The viral genome is 36Kb long and has been classified into 4 regions designated E1, E2, E3 and E4. Replication of adenovirus requires early expression of genes within the E1a region. Viruses defective in the E1a region have an insert capacity of up to 4Kb and although capable of infecting mammalian cells, are incapable of replication. Vectors that have had both the E1a and E4 regions removed have an insert capacity of almost 8Kb. However, replication defective virus can replicate in cells co-infected by wild type virus; cell lines genetically modified to express E1a genes and possibly cells infected by the Ebstein Barr virus (Horvath, Faxing, & Weber 1991). The human embryonic kidney 293 cell line (HEK293) contains the genetic sequence for the E1a region of the adenovirus serotype 5. This cell line has been used to propagate replication deficient adenoviral vector for use in *in vivo* experiments. Although replication deficient adenoviral vectors

seemed ideal for the planned experiments, other vector systems were considered. Liposome vectors had the attraction of being devoid of potentially antigenic viral proteins. However, the efficiency of transfection found with adenoviral vectors had not been matched by liposome vector systems. Adeno-associated virus could not be produced to the high titre possible with adenoviral vector. Retroviral vectors infect dividing cells and were not attractive candidates as vectors to non-dividing cardiac myocytes.

Group Experience

Dr Prentice and his group were experienced in propagating replication defective adenoviral vectors and had used these experimentally in cultured neonatal myocytes. In addition, shuttle plasmids encoding skeletal troponin C were under construction and these were suitable for insertion into commercially available adenoviral plasmids. It was anticipated that vectors co-expressing green fluorescent protein would allow identification of infected myocytes *in vivo*. Vector encoding mutants of skeletal troponin C with HA tags which were under construction had the potential to characterise the intracellular location of sTnC-HA within infected cells by use of appropriate antibodies and use of confocal microscopy. Given the group's experience, a decision was taken to utilise replication defective adenoviral vector in the *in vivo* rabbit experiments.

Replication Defective Adenoviral Vectors

The adenoviral vectors employed in the *in vivo* experiments were originally constructed by DOTAP or calcium precipitation transfection of 293 cells with a mixture of 40Kb long adenovirus plasmid DNA including the E1a region and shuttle plasmid containing the genetic sequence of interest, flanked on either side by sequences homologous to those flanking the E1a region of the large adenoviral plasmid. By a process of homologous recombination, the E1a region of the adenoviral plasmid is replaced by the gene of interest from the shuttle plasmid. The resulting adenoviral plasmid encodes the gene of interest, is E1a deficient and is within the limit to be packaged as an adenovirus within the complementing E1a expressing HEK293 cell line.

However, there is the possibility that such a system could produce replication competent virus, small enough to be packaged but still containing the E1a region. It is for this reason that vector produced by this method must be screened for replication competent virus. Where the process has produced a mixture of replication competent virus and replication defective adenoviral vector, the vector must be purified prior to use *in vivo.*

Viruses Employed

The first virus to be propagated encoded the gene beta-galactosidase (also known as *LacZ*) under the control of the ubiquitously expressing CMV promoter. This was a kind gift from Professor Ron Hay of the University of St Andrews. The vector had been constructed some years earlier, using the

plasmids available from Micobix (Microbix Inc, Toronto, Canada). The gene product is not known to have any specific effects on cardiac contractile function. Beta-galactosidase can metabolise X-gal to produce a blue dye and is commonly used as a reporter gene to identify cells that have been infected by vector. This vector had been propagated by the Howard Prentice group for some years and had been successfully employed in tissue culture experiments. Figure 3-1 is a typical *Hin*DIII restriction enzyme analysis of this vector.

*Hin*DIII specifically cleaves the sequence

5′... A^A G C T T ... 3′

3′... T T C G A^A ... 5′

to produce DNA fragments of differing lengths. The gel was run with a standard 1Kb ladder to help identification of specific fragments. This *Hin*DIII restriction enzyme pattern was similar to those produced by other group members when producing virus for use in cultured neonatal rat myocytes.

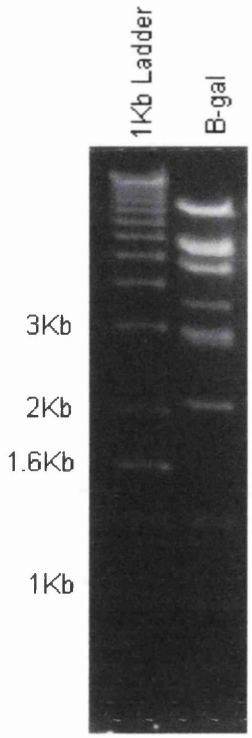


Figure 3.1. *Hin*DIII **restriction enzyme pattern of beta-galactosidase encoding adenovirus. Note double band at 3Kb.**

An aliquot of vector was propagated by the method previously described (Chapter 2, Page 71) and the resultant cellular lysate used for *in vivo* delivery to rabbit myocardium by the Swan-Ganz Catheter method (Chapter 2, Page 82). When an adult New Zealand white rabbit was exposed to $20x10^{10}$ pfu of this virus, it died within 2 days. At post mortem examination, an unexpected finding was of inflammation within the large airways and lungs. A second animal exposed to $21x10^{10}$ pfu of this virus died within one day. At post mortem examination, this animal had developed pleural effusions and congestion within the large airways. One possible explanation for these findings was contamination of the vector by replication competent wild type

virus. The wild type virus used in the lab was dl309. On restriction enzyme analysis with *Hin*DIII this virus has a characteristic doublet at 3Kb.

In vivo experiments were suspended, and further analysis of the vector was undertaken.

Possible Contamination by Wild Type Virus

Using primers specific for the E1a region of replication competent virus, a PCR analysis of the beta-galactosidase encoding vector was undertaken (Figure 3.2).

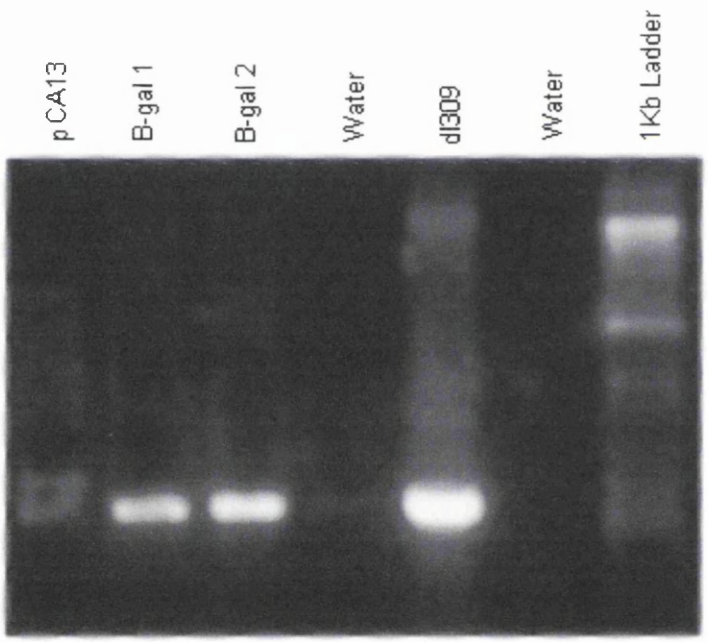


Figure 3.2. PCR using primers specific for E1a region of the adenovirus genome. pCA13 is a plasmid encoding the gene for beta-galactosidase. dl309 is a replication competent adenovirus and gave a strongly positive band. B-gal 1 was propagated from original seed stock and was thought to be replication deficient. B-gal 1 had been purified by caesium gradient prior to analysis. B-gal 2 was unpurified crude lysate. Both B-gal 1 and B-gal 2 gave strongly positive bands in keeping with the presence of the E1a region and by inference, replication competence.

Figure 3.3 is a *Hin*DIII restriction enzyme analysis of the replication competent virus dl309. The double band at 3Kb was similar to that found in the analysis of B-gal (Figure 3.1). On looking back through lab records when the original virus was gifted to Dr Prentice, this doublet was not detected.

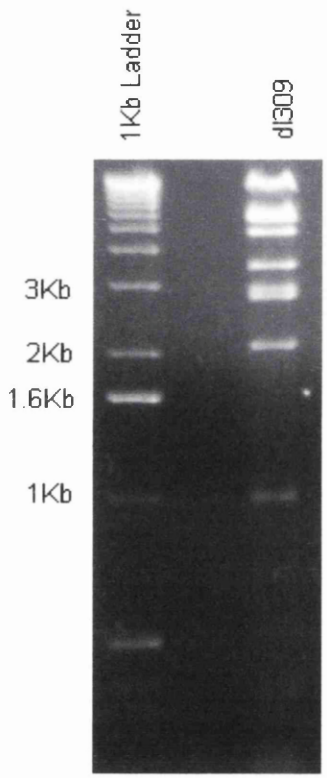


Figure 3.3. *Hin*DIII **restriction enzyme analysis of the wild type replication competent virus dl309.**

From the restriction enzyme analyses and PCR results, together with the early death of animals exposed to high doses of B-gal, it was concluded that the B-gal virus was contaminated by replication competent virus or was replication competent but also contained the sequence encoding beta-galactosidase.

*Hin*DIII restriction enzyme analyses were undertaken on viruses held by other group members. A typical result is shown (Figure 3.4).

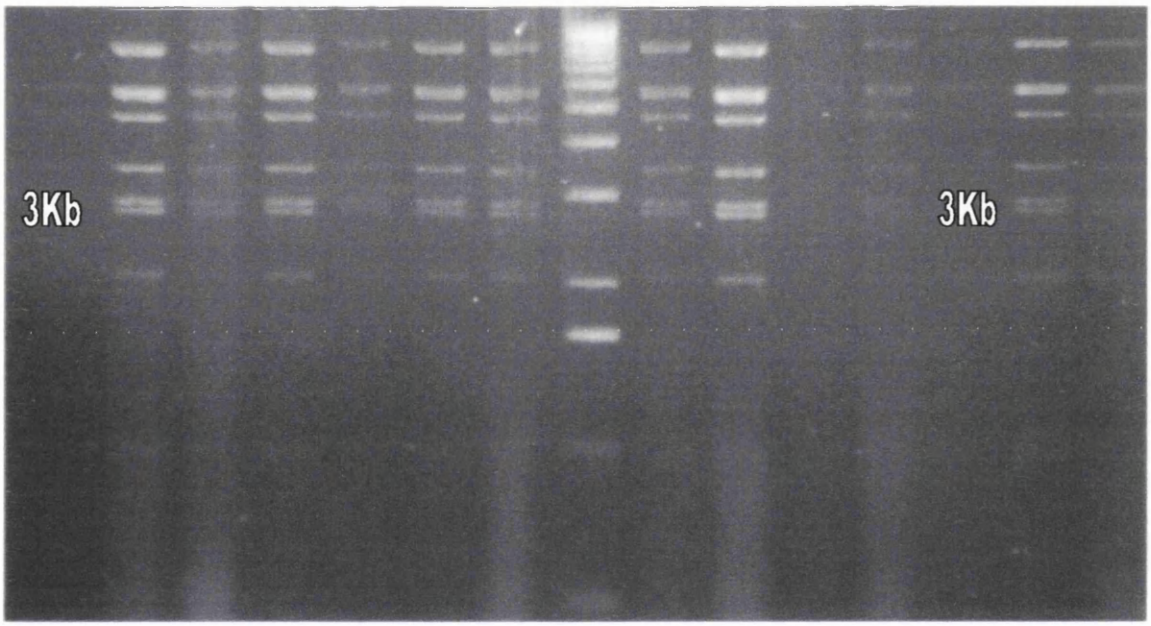


Figure 3.4 Typical result of *Hin*DIII restriction enzyme analysis of beta-galactosidase encoding viral vector held by other group members. Note the double band at 3Kb as previously found in the replication competent virus dl309.

All samples tested revealed a doublet band at 3Kb and it was concluded that all samples were potentially replication competent and not safe for use *in vivo*.

Plaque Purification of Contaminated Vector

The most likely explanation for the finding of replication competent adenovirus in all samples of the vector held by the group was that some time previously, the replication defective virus had been contaminated by wild type dl309 replication competent virus. The conclusion was that all samples of the vector encoding beta-galactosidase were a mixture of replication defective virus and replication competent virus. A decision was taken to isolate replication defective virus by a method of plaque purification. Serial dilutions of stock virus were used to infect dishes of HKE293 cells. A mixture of agar and growth medium was then carefully layered on top as previously described (Chapter 2, Page 76). Following up to seven days of incubation, dishes with fewer than ten plaques were selected. Individual plaques, isolated using sterile Pasteur pipettes, were used to infect dishes of HKE293 cells. After three or more days of incubation, the cells from infected tissues were harvested and the resulting crude lysate was characterised by *Hin*DIII restriction enzyme analysis. Figure 3.5 is representative of a typical result. Over 100 individual plaques were grown, carefully isolated to avoid cross-contamination, and used to infect dishes of HKE293 cells. Each was investigated by restriction enzyme analysis. Despite this time-consuming process, none of the virus isolated was free of the double band at 3Kb, indicative of contamination by wild type, replication competent virus.

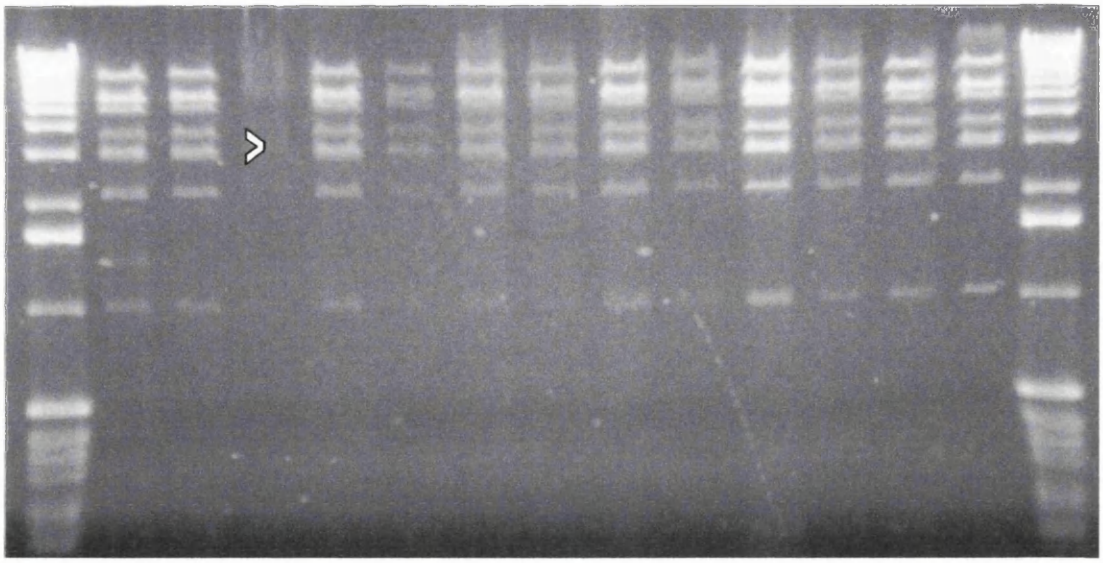


Figure 3.5. 1Kb ladder in first and last lane. All other lanes show result of *Hin*DIII restriction enzyme analysis of virus propagated from isolated viral plaques. Arrow (>) at 3Kb with double band detected in all samples, indicative of contamination by replication competent virus.

Purification of Contaminated Vector by Serial Dilution

Twenty-four well tissue culture dishes were used to grow 293 cells to a confluency of 50%. Following serial dilution of contaminated virus, wells were infected and propagated for up to one week. This method failed to isolate replication defective virus encoding beta-galactosidase.

Fresh Stock Vector Obtained

Fresh stock virus was obtained from Prof Ron Hays, University of St. Andrews. Following serial dilution, six discrete plaques were isolated from dishes overlayed with agar/growth medium mixture. Three plaques were

isolated on day 6 and a further three plaques isolated on day 8. Of these six plaques, the crude lysate grown from the 3 plaques picked on day 8 produced a blue dye when mixed with X-gal, indicating expression of beta-galactosidase. The crude lysate grown from the 3 plaques picked on day 6 failed to produce a blue dye when mixed with X-gal, indicating that beta-galactosidase had not been expressed. The *Hin*DIII restriction enzyme analysis of crude lysate grown from the beta-galactosidase expressing plaques is shown in Figure 3.6.

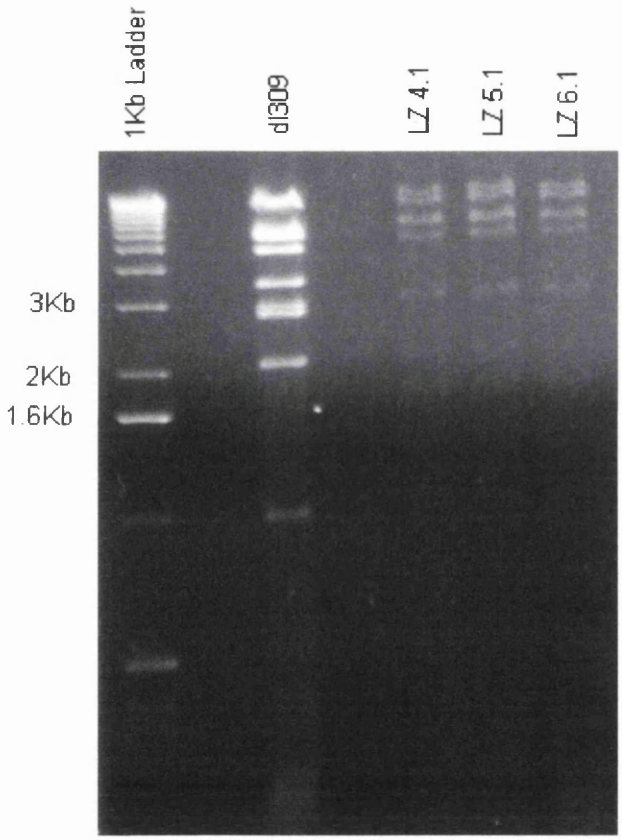


Figure 3.6. *Hin*DIII **restriction enzyme analysis of crude lysate grown from 3 plaques (LZ4.1, LZ5.1, LZ6.1) isolated from new stock as gifted by Prof Ron Hays, University of St. Andrews. Note the double band at 3Kb found in the replication competent dl309 virus was not detected in the plaques isolated from the new stock virus encoding beta-galactosidase.**

Importantly, there was no double band detectable at 3Kb in any of the three isolates analysed. These were thought suitable for use in *in vivo* experiments and were used as seed stock for viral propagations.

A PCR analysis was undertaken to confirm the presence on the beta-galactosidase DNA sequence and exclude the presence of the E1a DNA sequence from the replication defective viruses.

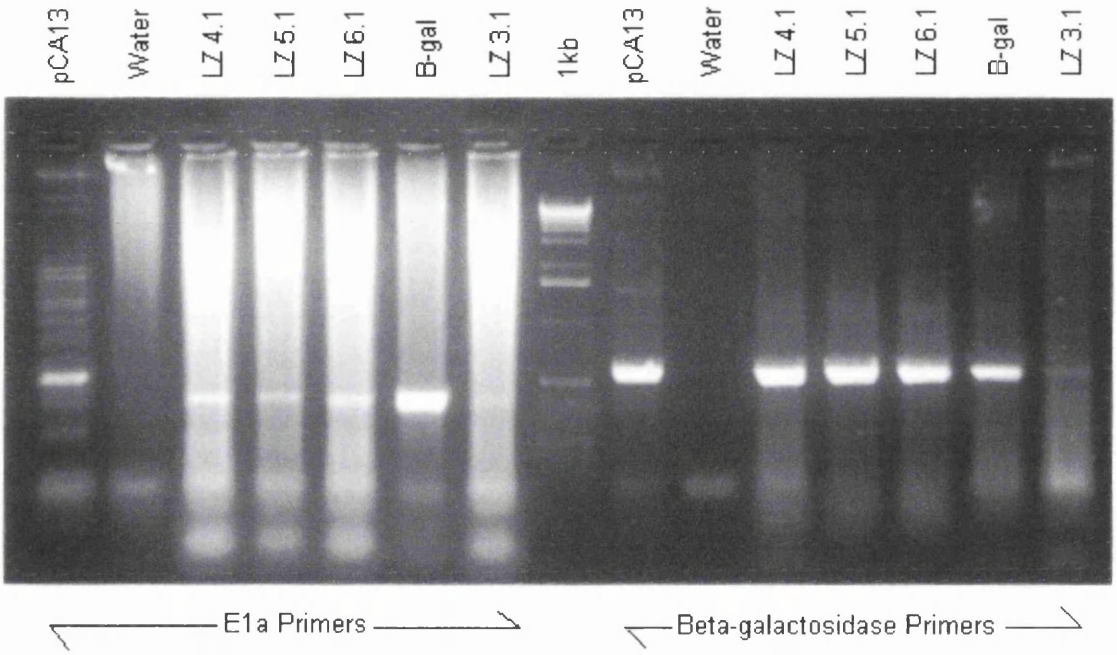


Figure 3-7 PCR of pCA13 (plasmid encoding the sequence for beta-galactosidase), Distilled water, LZ 4.1, 5.1, 6.1 (Plaques purified from new seed stock from University of St Andrews, the crude lysate of which produced a blue dye when incubated with X-gal), B-gal (Original stock virus, thought to be contaminated with replication competent virus), LZ 3.1 (plaque purified from new seed stock from University of St Andrews but did not produce a blue dye when incubated with X-gal), 1Kb (standard 1Kb DNA ladder). The PCR performed with the E1a primers was not undertaken with a standard "hot start" which may explain some of the non-specific smearing.

A strongly positive signal for E1a was found with the original B-gal virus as would be expected with contamination by wild type virus. No signal was found in the distilled water blank indicating that the PCR reagents were not contaminated. Non-specific banding was found in the pCA13 lane in keeping with non-specific binding associated with a cold start PCR. Faint bands were detected at 416bp in LZ 4.1, 5.1, 6.1 and LZ 3.1. These bands could represent either contamination of these plaque-purified isolates by replication competent virus or amplification of E1a region DNA carried from the 293 cells in which the virus was propagated.

The PCR using primers for the Beta-galactosidase sequence gave positive signals for the positive control pCA13, negative signal for the water blank and strongly positive signal for the viral isolates that had produced a blue dye when incubated with X-gal. LZ 3.1 gave only a very weak signal for beta-galactosidase. These results indicate that the new seed stock from the University of St Andrews was a mixture of virus encoding for beta-galactosidase and virus not encoding for beta-galactosidase. The *Hin*DIII restriction enzyme analysis of LZ 4.1, 5.1 and 6.1 did not detect a double band at 3Kb suggesting that these isolates were free of contamination by replication competent virus. However, although the weak PCR signal for the presence of E1a in these samples could be explained by carry over of 293 cell DNA containing E1a, the possibility of some contamination of these isolates by replication competent virus remained.

The issue of possible low-level contamination of adenoviral vector by replication competent virus can be addressed by amplification of replication competent virus in cell lines that are permissive to replication competent virus

but non-permissive to replication defective virus. Such a method has been developed and has been termed the "Supernatant rescue assay" (Dion, Fang, & Garver, Jr. 1996). Such methods can detect one replication competent particle in a mixture of 10^9 replication defective particles. Sensitive methods such as these are mandatory for vector intended for use in clinical trials. For the purpose of the experiments intended in this project, a pragmatic decision was taken that virus with a satisfactory *Hin*DIII restriction enzyme pattern could be used within the confines of the laboratory with the precaution that all potentially contaminated instruments be autoclaved and any waste products incinerated.

Rates of Viral Replication

Why was it not possible to isolate replication deficient adenovirus from the original beta-galactosidase encoding vector held in the lab? If the fresh stock of virus from the University of St Andrews contained a mixture of virus expressing and not expressing beta-galactosidase, why did the non-expressing virus produce plaques 2 days before the beta-galactosidase expressing virus? The answer to both of these questions may in part be explained by a property of adenoviral vectors first described by Bett et al in 1993 (Bett, Prevec, & Graham 1993). Adenovirus type 5 can package genome sizes up to 108% of wild type genome length. However, Bett et al found that vector consisting of 108% of the wild type genome had a replication rate of half that of wild type virus. This property of smaller genomic sized viruses replicating more quickly than larger genomic size vectors would explain why it proved difficult to isolate non-wild type virus from the original heavily

contaminated beta-galactosidase encoding stock vector. The wild type plaques may have developed and spread across the layer of HEK293 cells before the replication defective virus plaques could become apparent. Similarly, when the fresh vector from the University of St Andrews was used to produce plaques, the virus that did not express beta-galactosidase (potentially smaller genome) produced plaques 2 days ahead of the virus that did express beta-galactosidase.

Adenoviral Vector Encoding Nuclear-localising Beta-galactosidase

Dr Hajjar of Harvard Medical School, Boston, USA kindly gifted a replication defective adenoviral vector encoding a nuclear-localising beta-galactosidase under the control of the RSV (Rous sarcoma virus) promoter. The method of construction had been published, and this virus has been successfully used *in vivo* in the rat (Dong, Schulick, DeYoung, & Dichek 1996; Hajjar et al. 1998) . *Hin*DIII restriction enzyme analyses of virus propagated from single plaques were performed. Reassuringly, there was no detectable double band at 3Kb (See Figure 3.8).

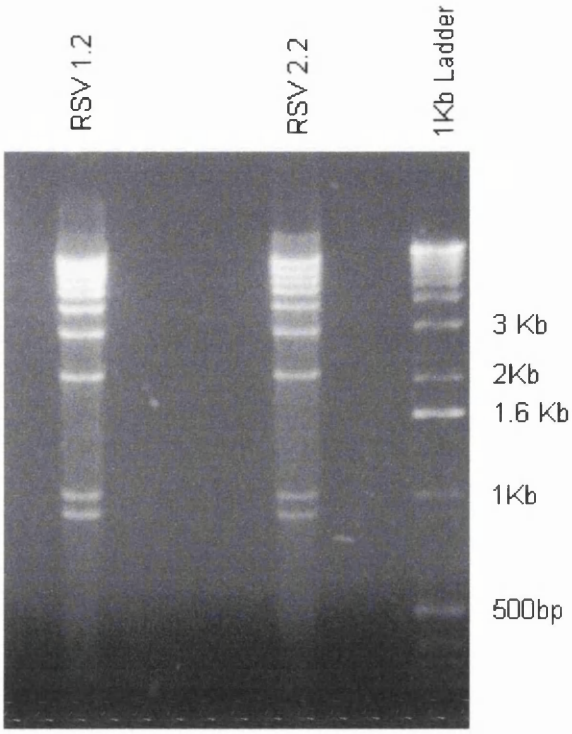


Figure 3.8. ***Hin*DIII restriction enzyme analysis of replication deficient virus encoding nuclear-localising beta-galactosidase under the control of the RSV promoter. RSV 1.2, 2.2 were propagated from two isolated plaques.**

Adenoviral Vectors Encoding Skeletal Troponin C

Dr Obeid Khan had constructed replication defective adenoviral vector encoding skeletal troponin C under the supervision of Dr Prentice (Khan 1999). The *Hin*DIII restriction enzyme pattern for the various constructs was known. Figure 3-9 demonstrates the *Hin*DIII restriction enzyme patterns for sTnC HA HCA and sTnC HA CMV. These were found to be similar to those produced by Dr Khan and these isolates were used to propagate virus for *in vivo* experiments. In Figure 3-9, RSVLZ1.3 was the *Hin*DIII restriction enzyme pattern of nuclear-localising beta-galactosidase encoding adenoviral vector gifted by Dr Hajjar. RSVLZ1.3.2 and RSVLZ1.3.3 were the *Hin*DIII restriction enzyme patterns of virus produced by propagation of RSVLZ1.3 and were

found to be similar. These viruses were subsequently used in *in vivo* experiments.

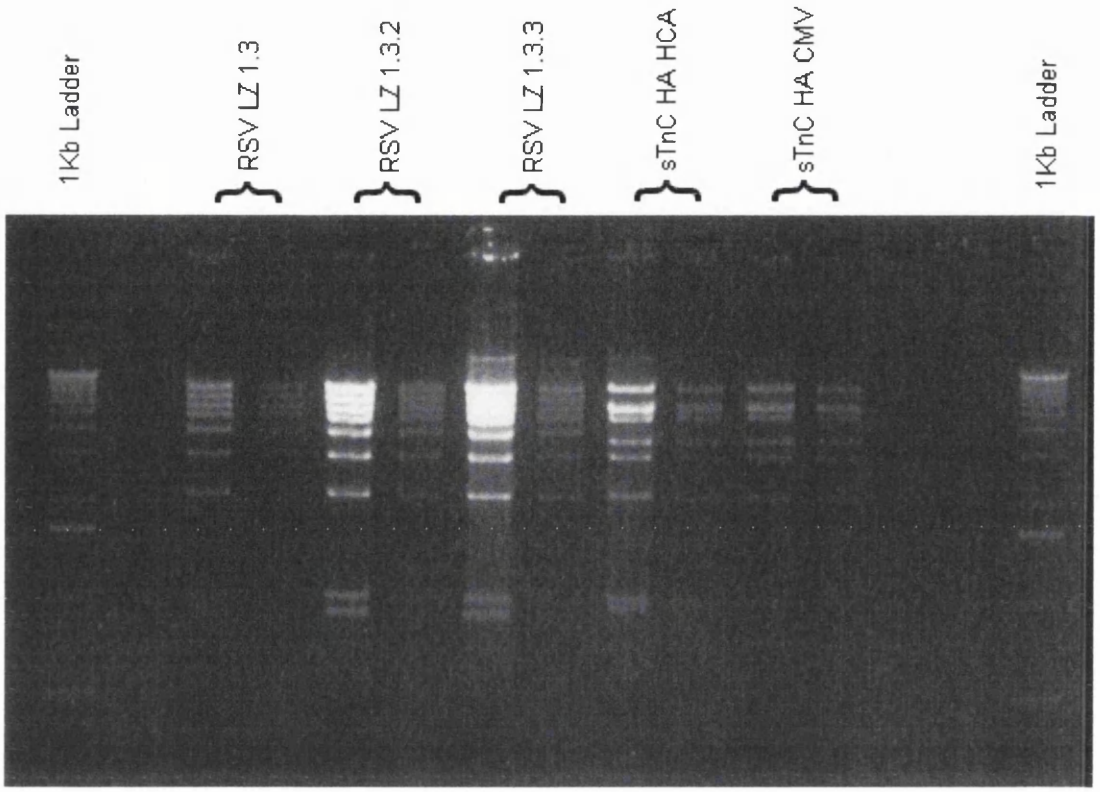


Figure 3.9. *Hin*DIII restriction enzyme patterns of viral vectors subsequently used in *in vivo* experiments. With the exception of RSV LZ 1.3, all viruses had been concentrated and purified using a caesium chloride gradient. The right hand lane for each virus was a 1:10 dilution of the left hand lane. No doublet at 3Kb found in any of these viruses.

Discussion

Contamination of the original beta-galactosidase expressing adenoviral vector stock by replication competent wild type virus may have contributed to the inflammatory reaction in the respiratory tract of the rabbits exposed to

$20x10^{10}$ pfu of virus. The data from the *Hin*DIII restriction enzyme analyses support this conclusion. Cellular debris and antigenic proteins from the crude lysate may have contributed to the inflammatory reaction. Although PCR was used to confirm the presence of E1a DNA in the lysate from contaminated plaques, this in retrospect was not informative since carry over of HEK293 cell DNA encoding E1a cannot be excluded. Dion et al have developed a sensitive bioassay method of detecting *viral* E1a DNA (Dion, Fang, & Garver, Jr.1996). This requires serial propagation through HeLa cells. Even this method does not exclude wild type contamination completely, the detection limit being one replication competent viron in 10^9 replication deficient virons.

Conclusions

Viral lysate contaminated with wild type replication competent virus is unsuitable for *in vivo* use. The most sensitive method of detecting replication competent contamination is by the supernatant rescue bioassay method (Dion, Fang, & Garver, Jr. 1996). The sensitive bioassay method for detecting replication competent virus is more sensitive than PCR methods but has a detection threshold of 1 in 10^9 and therefore contamination by replication competent virus can never be excluded. For *in vivo* animal experiments we used vector propagated from single isolated plaques, concentrated and purified of cellular debris on a caesium chloride gradient, and with no evidence of wild type contamination on DNA restriction enzyme analysis. However, the possibility remained that there was wild type contamination of virus despite these measures.

RESULTS 4

Development of the Swan-Ganz Method of Vector Delivery

Introduction

Studies on single cardiac myocytes isolated from transgenic mice expressing sTnC have reported a relative resistance to the calcium desensitising effects of intracellular acidosis (Metzger et al. 1993). Acidosis is only one component of ischaemia and although cells expressing sTnC may remain responsive to the calcium transient in conditions of acidosis, there may be other components of ischaemia that might limit the potentially beneficial effects of sTnC expression in the heart. In order to test the hypothesis that adenoviral gene transfer of sTnC to rabbit myocardium would result in a desirable physiological change, an effective method of delivering viral vector to the intact heart was required. Although components of ischaemia can be tested in isolated myocytes and trabeculae, our aim was to investigate the effect of ischaemia on whole heart preparations in which a large proportion of myocytes expressed a foreign gene of interest. Coronary occlusion can be used in anaesthetised whole animal preparations to study the effects of true ischaemia (Bauer et al. 1993). Similarly, coronary ligation can be used in isolated working heart preparations. However, in working heart preparations, metabolism is switched from fatty acid utilisation to carbohydrate utilisation. In order to use whole animal or working heart preparations a method of viral delivery was required which would maximise the proportion of myocytes infected and minimise any traumatic or inflammatory process. It was anticipated that 5 to 7 days would be required following viral introduction for optimal gene expression (Guzman et al. 1993). Barr et al had described an efficient method of viral delivery (Barr et al. 1994). This group employed selective coronary catheterisation to deliver adenoviral vector directly into one of the coronary arteries. Using this method, up to 32% of the ventricular

cardiac myocytes within the distribution of a single coronary artery display foreign gene expression. We wished to develop a catheter based method of delivering adenoviral vector simultaneously down both left and right coronary arteries, allowing multiple injections without risking mechanical trauma to the coronary arteries. It was hoped that multiple injections of viral vector may result in a proportion of infected cardiac myocytes greater than that produced by Barr et al. Adenoviral gene transfer to myocardium had been reported using direct needle injection (Guzman et al. 1993). Although this method reliably infects cells around the injection site, it results in trauma and inflammation around the needle tract. In rabbits, direct needle injection would require open thoracotomy and was an unattractive method of achieving transfection of the relatively thin right ventricle (risk of rupture) and inaccessible intra-ventricular septum.

Development of a Swan-Ganz Catheter Mediated Vector Delivery

There had been no prior reports of using a Swan-Ganz catheter to deliver adenoviral vector to the coronary circulation. The Swan-Ganz method developed here was relatively non-invasive and did not require a thoracotomy (Chapter 2, Page 82). Table 4.1 summaries the procedural results of the preliminary experiments.

Table 4.1. Procedural Results of Swan-Ganz Experiments

Rabbit ID and Weight	Procedural Details	Outcome
Rabbit 96/736 3.1Kg	Unable to occlude aorta with 5F balloon 7F very difficult to pass but balloon occludes ascending aorta Able to tolerate 30sec inflation.	Died on 90sec inflation.
Rabbit 96/712 3.7Kg	Able to pass 7F catheter and occlude aorta. Tolerated one 30sec inflation and one 10 sec inflation. Injected $3x10^8$ pfu CMV LacZ Ad5 lysate in a volume of 9 ml Tris Saline	Sacrificed day 6
Rabbit 96/738 3.3Kg	Unable to pass 7F catheter on lighter animal. Able to pass 6F catheter, 1.5ml balloon occluded aorta. Injected 2x3ml of $1x10^9$ pfu/ml CMV LacZ Ad5 lysate, 30sec inflation with each 3ml injection.	Rupture carotid on withdrawal of catheter. Died
Rabbit 96/740 3.3Kg	Able to pass 6F catheter and occlude aorta. Injected 12 ml of $5x10^8$ pfu CMV LacZ Ad5 lysate as 4 X 3ml injections. Each injection with 15-20 sec inflation.	Sacrificed day 6
Rabbit 96/769 3.2Kg	Able to pass 6F catheter and occlude aorta. Injected 8 ml of $1x10^9$ pfu/ml CMV LacZ Ad5 lysate as 8 x 1 ml. Each injection with a 30 second balloon inflation.	Heart progressively larger, died on last inflation.
Rabbit 96/784 3.4Kg	Able to pass 6F catheter and occlude aorta. Injected 22ml of 5 x 10^9 pfu/ml CMV LacZ Ad5 lysate as 22 x 1ml injection, 10sec balloon inflation with each. 3 min recovery between injections. Intra-aortic BP monitoring satisfactory	Sacrificed day 6
Rabbit97/849 2.9Kg	Unable to pass 6F catheter.	On passing 5F, fatal leakage of carotid artery.

Procedural Results

A 3.2Kg New Zealand white rabbit could be reliably cannulated using a 6F Swan-Ganz catheter and the aorta occluded by inflating the 1.5ml balloon. Rabbits under 3.0Kg could have their carotid arteries cannulated with a 5F Swan-Ganz catheter. However, the smaller catheter had a correspondingly smaller balloon, making occlusion of the ascending aorta difficult. Occlusion of the ascending aorta was thought important in preventing spillage of virus into the systemic arterial system and restricting the flow of virus injected below the balloon to the coronary circulation. Repeated short inflations were well tolerated but prolonged inflations of 30 seconds or more were associated with procedural deaths.

Histological Results 1

Rabbit 96/712 was the first animal to survive the Swan-Ganz catheter method of delivery. This was a relatively large animal that had a carotid artery large enough to pass a 7F Swan-Ganz catheter and achieve occlusion of the ascending aorta on inflation of the balloon. Only $3x10^8$ pfu of virus encoding beta-galactosidase was used, one order of magnitude less than that used by Barr et al. Six days following viral introduction, the animal was sacrificed and the heart taken fresh for histological staining. The method of staining this whole heart was similar to that used by other group members in staining infected neonatal cardiac myocytes (Chapter 2, Page 86, Method 1). After 24 hours of incubation in X-gal solution the whole heart appeared dark blue,

almost black. This preliminary result was surprising and initially suggested very efficient transfection of a large proportion of the myocardium with a relatively small amount of viral vector. However, on histological sectioning it became apparent that the dark pigmentation was restricted to a thin coating on the epicardial surface and that no blue staining was apparent in the myocytes. There was no negative control tissue stained concurrently with this heart and a false positive result was suspected.

Specificity of Staining Method 1

To test the specificity of the staining method, direct needle injection of adenoviral vector was attempted in rats and then rabbits (Chapter 2, Page 82). Direct needle injection was chosen as a method of guaranteeing that some cells around the needle tract would be infected. The procedural details are tabulated (Table 4.2). Two rats died immediately after injection of high concentration adenoviral vector. However, direct needle injections were successfully performed on two rabbits using low concentration of virus. Each heart was injected with a quantity of stock virus and in a separate area, virus propagated from stock virus. The hearts were harvested seven days following the introduction of viral vector.

Table 4.2. Procedural Details of Direct Needle Injection Experiments

Animal ID and Weight	Procedural Details
Rat 51/96u 500g	200μl of 10^{10} pfu/ml CMV LacZ Ad5 crude lysate. Asystolic, died on table.
Rat 52/96b 500g	200μl of 10^{10} pfu/ml CMV LacZ Ad5 crude lysate. Asystolic, died on table.
Rabbit 774 3.4Kg	100μl of $5x10^{8}$ pfu/ml CMV LacZ Ad5 crude lysate to apex. 100μl of 1:10 dilution of Lab Stock CMV LacZ Ad5
Rabbit 750 3.4Kg	100μl of $5x10^{8}$ pfu/ml CMV LacZ Ad5 crude lysate to apex. 100μl of 1:10 dilution of Lab Stock CMV LacZ Ad5

Histological Results 2

The tissue from rabbit 96/712 had been stained using stock solutions used by the group for staining rat neonatal cardiac myocytes. Fresh solutions were used to stain tissue from the direct needle injection animals. The fixative was changed to 10 minutes of 4% paraformaldehyde as this was thought less likely to denature the beta-galactosidase enzyme. Three blocks of tissue were cut from each heart. These consisted of the two injected areas and an un-injected area. Each block was halved, with one half being stained immediately and the other sent for frozen sectioning by Niall Whyte, Department of Pathology, Western Infirmary, Glasgow. The frozen sections were stained the same day with the same solutions. All tissue samples were incubation in X-gal solution for 3 hours. No counter-stain was used for fear of washing out the blue pigment from tissue expressing beta-galactosidase. Tissue blocks, which

had been stained immediately, were sent the same day for processing and sectioning from wax blocks.

No blue staining was detected in un-injected areas. Blue staining was evident in the tissues that had been injected with vector encoding beta-galactosidase. The sections stained immediately and subsequently cut from wax blocks displayed blue staining on the periphery of the injection site (Plate 4.1.). Direct needle injection is known to cause trauma and inflammation and it was not surprising that the centre of the injection area did not stain positively for beta-galactosidase expression. A similar appearance was found in the frozen sections. On examination of the frozen sections of rabbit 96/774 there seemed to be more staining more centrally (Plate 4.2). Since no counter-stain was used, the morphology of positively staining cells could not be determined.

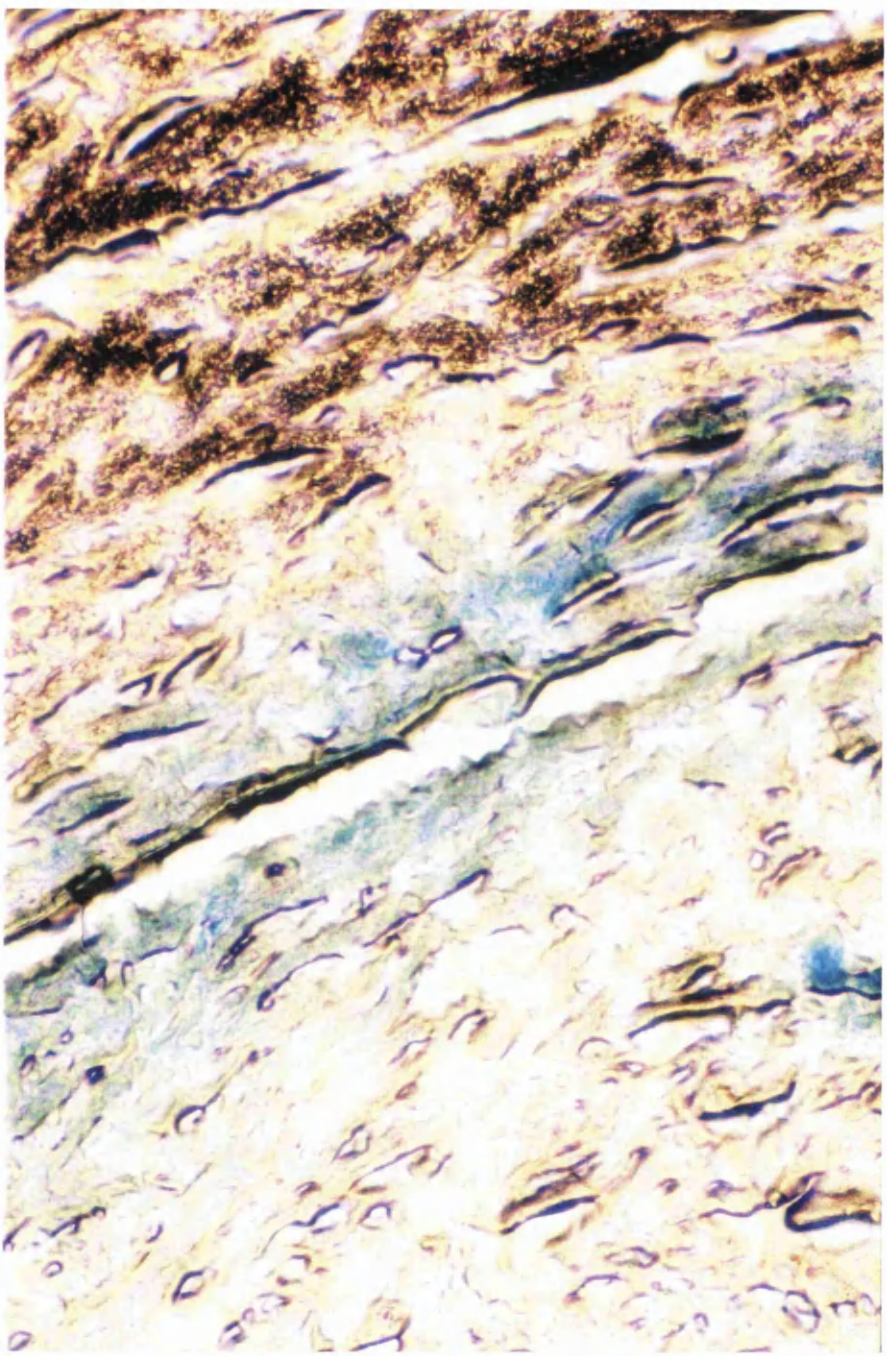


Plate 4.1 An example of a histological section (magnification X40) of rabbit myocardium 7 days following direct needle injection of vector encoding beta-galactosidase. Some blue staining is visible on the periphery of the injection site. No counter-stain was used.

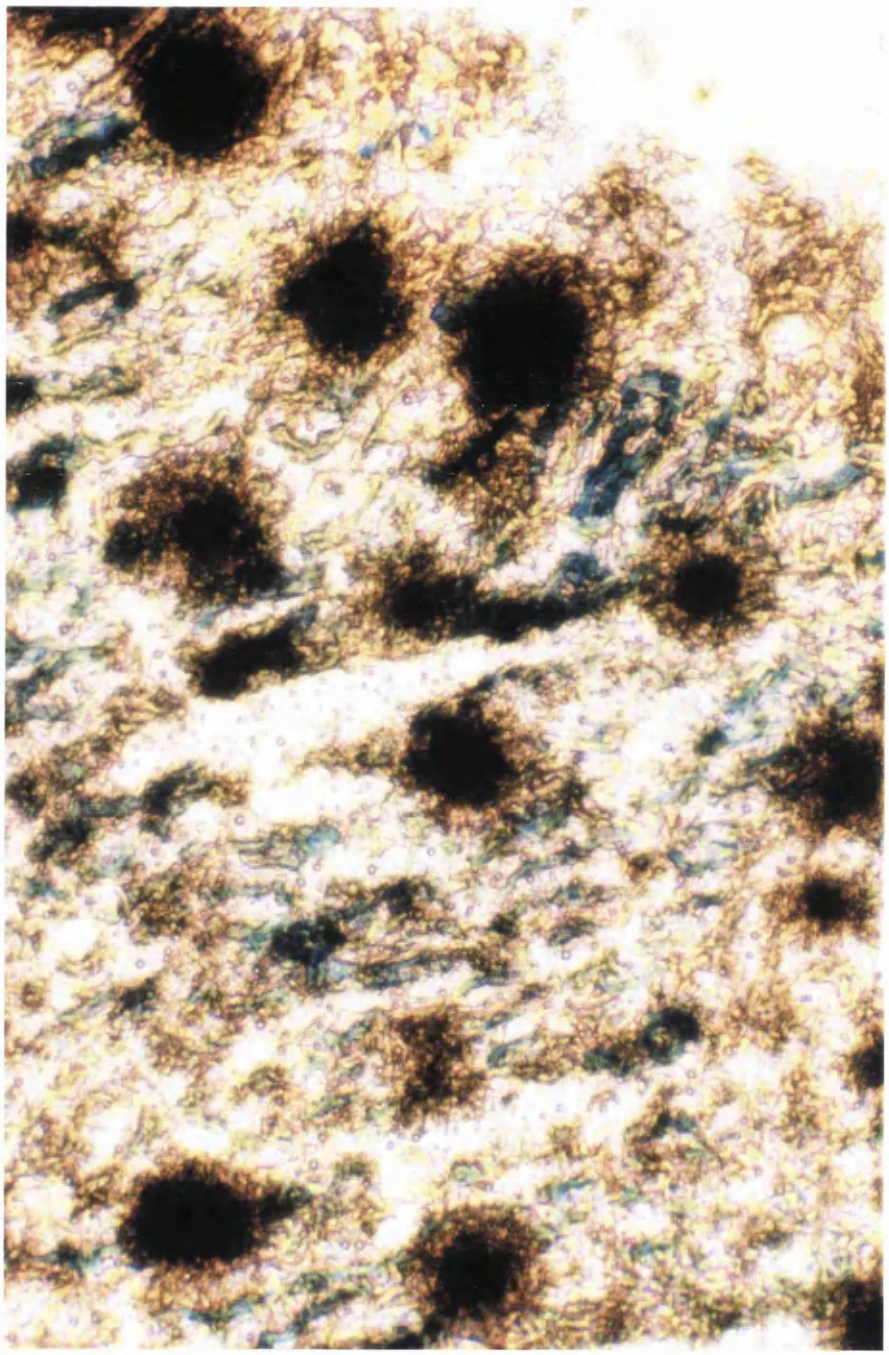


Plate 4.2 An example of a histological section (magnification X25) of rabbit myocardium 7 days following direct needle injection of vector encoding beta-galactosidase. Some blue staining is visible on the periphery of the injection site. No counter-stain was used.

Discussion of Direct Needle Injection Experiments.

The purpose of these experiments was to ensure the specificity of the modified staining method used to stain tissue expressing beta-galactosidase. The use of the uninjected control in the direct needle injection experiments suggested that the staining method used was specific. In retrospect, better negative controls would have been direct needle injection using saline and a separate group injected with empty virus. This is because subsequently published data in which liposomes not encoding beta-galactosidase were injected into the coronary circulation of rabbits resulted in blue staining of the myocardium on exposure to X-gal (Wright et al. 1998). Using their staining method, blue staining was noted in damaged myocardium without there having been delivery of an exogenous beta-galactosidase gene. Therefore, from the direct needle injection data alone, the possibility of false positive staining secondary to myocardial damage could not be excluded. X-gal is an indole derivative attached to a carbohydrate molecule Beta-galactosidase cleaves the X-gal molecule, releasing an indoxyl monomer. The monomers can become dimeric by a process of non-enzymatic oxidation. A ferro-ferri-cyanide redox buffer is commonly used to promote the dimerisation of the monomers, producing a water insoluble blue dye. However, in acidic conditions, the iron from the ferro-ferri-cyanide redox buffer can precipitate out of solution, coating tissues with a dark blue, almost black pigment (Prussian Blue). This may be the mechanism by which false positive staining occurred in the first rabbit to survive the Swan-Ganz delivery method. This false positive staining can be avoided by the use of pH buffers or copper-cyanide (which

gives a green precipitate, distinguishable from true staining of beta-galactosidase expressing tissue). The hearts from animals 96/740 and 96/784 had been frozen for later analysis. On defrosting and staining of these hearts, no evidence of beta-galactosidase expression was detected.

Conclusion of Direct Needle Injection Experiments

It was felt that the staining method was sufficiently specific to proceed with dose finding experiments employing the Swan-Ganz viral delivery method. However, we did not include control animals where only vehicle was injected. It was subsequently reported that false positive staining can occur in areas of micro-infarction within rabbit myocardium (Wright et al. 1998). In retrospect, myocardium injected with vehicle alone should have been included as a negative control. In addition, a third group injected with empty virus would have controlled for the possibility that adenovirus in itself could cause false positive staining.

Dose Finding Study (Swan-Ganz Delivery Method)

In total seven rabbits were exposed to between sixteen and 20 ml of adenoviral vector encoding beta-galactosidase. The concentration of virus use varied from $5x10^9$ pfu/ml to $4.2x10^{10}$ pfu/ml. The procedural and histological results are tabulated (Table 4-3).

Table 4-3. Dose Finding Study (Unfiltered Crude Lysate)

Rabbit ID	Weight (Kg)	Cath size	Viral pfu/ml Conc	Injections	Sacrifice at Day	Procedural and Histological Results
97/892	3.3	6f	$5x10^9$	17	6	Balloon failure, no B-gal stain
97/904	**3.0**	**6f**	**$6.5x10^9$**	**17**	**6**	**Patchy reporter gene expression in association with vessels. Inflammatory response.**
97/909	3.3	7f/6f	Sham	None	6	No staining in negative control
97/950	**3.4**	**7f**	**$7.5x10^9$**	**20**	**6**	**Unexpectedly, no reporter gene expression**
97/915	3.2	6f	$8.75x10^9$	16	Peri-operative death	Catheter pushed against aortic valve, no pressure measurement possible.
97/953	3.0	6f	$10x10^9$	20	Peri-operative death	Left sided limb weakness in recovery
97/962	**3.3**	**7f**	**$11x10^9$**	**20**	**Died day 2**	**Died after 2 days. Inflammation in trachea. Bilateral pleural effusions. Peri-vascular reporter gene expression in RV.**
97/1065	3.6	7f	$42x10^9$	19 filtered	Died < 12hr	No histology as contamination with wild type virus suspected.

Only three of the six animals exposed to the viral vector survived to day six. These animals received relatively low concentrations of virus and positive staining for the presence of beta-galactosidase was apparent in animal 97/904 only. Animal 97/915 died during the procedure. To ensure occlusion of the ascending aorta, in this particular animal, the catheter had to be pushed against the aortic valve. Because of this it proved impossible to measure the aortic pressure during balloon inflations. This animal's death was recorded as procedural in nature. Similarly, the left-sided weakness found in animal 97/953 following instrumentation of the right carotid artery was recorded as a procedural complication and the animal was immediately euthanised.

The early deaths of animals 97/962 and 97/1065 were not thought to have been procedural in aetiology. At post-mortem examination both animals had evidence of gross congestion and inflammation within their respiratory system. Perivascular regions within the right ventricle of animal 97/962 stained positively for the reporter gene product beta-galactosidase. Contamination of the replication deficient adenoviral vector by wild type virus was suspected and for reasons of safety, the heart of animal 97/1065 was not taken for histology. Restriction enzyme analysis of the viral vector and stock virus used subsequently confirmed contamination by wild-type virus dl309 (Chapter 3, Page 108)

Discussion of Swan-Ganz Viral Vector Delivery Method

It was encouraging that using the Swan-Ganz delivery method, at least two animals displayed evidence of foreign gene expression. However, when compared with the results obtained by Barr et al using selective coronary injection, the Swan-Ganz method of delivery appeared disappointingly inefficient. In at least two animals more virus was used than in any of Barr's selective coronary injection animals and no evidence of gene transfer was found. During the time taken to analyse and attempt to purify the contaminated stocks of original adenoviral vector encoding beta-galactosidase, Donahue et al published their findings on factors determining adenoviral mediated gene transfer to rabbit myocardium *in vitro* (Donahue et al. 1997). In tissue culture experiments, Donahue found that mixing adenoviral vector with whole blood, but not sera, reduced the efficiency of transfection. The conclusion was that the presence of red blood cells reduced the efficiency of transfection. Similarly, mixing vector with iodine containing radio-opaque contrast material reduced the efficiency of viral infection in tissue culture. Using a re-circulating Langendorff isolated heart preparation, Donahue et al demonstrated that long exposure time, high viral concentration and high coronary flow were factors favouring efficient uptake of viral vector in the intact heart. Given this new information, the Swan-Ganz method had several potential flaws. The dead space between the aortic valve and the inflated balloon of the Swan-Ganz catheter provided a potential space for mixing of adenoviral vector and red blood cells from the left ventricle. In addition, there was

the possibility of exposing the vector to radio-opaque contrast injected to confirm satisfactory balloon inflation. The mixing of red blood cells and contrast material with the adenoviral vector may have reduced the efficiency of gene transfer to the myocardium. Coronary artery flow occurs during diastole when the aortic pressure exceeds trans-myocardial pressure. The rate of coronary artery flow during diastole is related to the difference in pressure between the aorta and the left ventricular cavity. It is possible that with the ascending aorta completely occluded by the Swan–Ganz balloon, the pressure in the left ventricular cavity would increase, limiting coronary artery flow. This was a further potential disadvantage of the Swan-Ganz method. The main theoretical advantage of using a Swan-Ganz catheter was the potential for multiple simultaneous exposures of both the coronary arteries to vector without risking trauma from use of a selective catheter. These preliminary results suggested that the Swan-Ganz catheter method of delivery was substantially inferior to Barr's selective coronary injection method. Given the labour-intensive and time-consuming nature of adenoviral propagation, this made the Swan-Ganz method very unattractive.

Conclusions

In the adult New Zealand white rabbit it is possible to deliver adenoviral vector to the coronary circulation using a Swan-Ganz catheter. Given the limitations of the histological methods used it was not possible to confirm that cardiac myocytes had been infected. The discovery that the vector

used was heavily contaminated with replication competent virus made comparison of the efficiency of this method with a historical selective coronary injection method impossible. In addition, the supernatant from crude lysate was used and this would have included potentially antigenic material from the human embryonic kidney cell line in which the vector had been propagated. This may have contributed to the inflammatory reaction seen in some animals. The volumes of virus used approached 10% body weight and it is likely that larger volumes would be poorly tolerated and may have resulted in pulmonary oedema. It is possible that the theoretical advantage of multiple injections using the Swan-Ganz catheter would be offset by disadvantageous changes in coronary artery flow rate during balloon inflations. In addition, the data presented by Donahue et al that mixing of the adenoviral vector with blood and radio-opaque contrast can reduce the efficiency of gene transfer to isolated myocytes, made the Swan-Ganz catheter method an unattractive candidate for further development.

CHAPTER 5

Development of a Selective Coronary Catheterisation Method of Vector Delivery

Introduction

An *in vivo* selective coronary injection method of adenoviral mediated gene transfer to rabbit myocardium was first published some four years before the experiments detailed in this chapter (Barr et al. 1994). In that time no other group had published using this method. It was anticipated that development of a selective coronary injection method of adenoviral delivery would be problematic. Selective coronary injection required custom-made catheters suitable for use in rabbits and fluoroscopic screening equipment with adequate resolution to visualise rabbit coronary arteries. Anticipated complications included bleeding at the carotid artery vascular entry site, damage to the ascending aorta, and damage to the instrumented coronary artery itself. However, the method of selective coronary injection had theoretical advantages over the Swan-Ganz method (Chapter 2, Page 84). By plugging the coronary artery with the catheter and transiently isolating the coronary artery from the aorta, there was the possibility of preventing viral vector from mixing with red blood cells. In addition, selective coronary injection had the potential of controlling the rate of flow of vector down the coronary artery. Exclusion of red blood cells and optimal coronary flow had been found to be important determinants of efficiency of adenoviral mediated gene transfer *in vitro* (Donahue et al. 1997). Given the labour intensive and time-consuming nature of adenoviral vector production, an efficient method of vector delivery was desirable.

The aim of these preliminary selective coronary injection experiments was to assess the feasibility of causing foreign gene expression in rabbit cardiomyocytes by selective coronary injection of replication deficient adenoviral vector.

Methods

Either the left or the right coronary artery was selectively cannulated and adenoviral vector injected as previously described (Chapter 2, Page 84). A custom-made 3 French nylon coronary catheter with a hockey-stick tip (Royal Flush II Radiopaque Nylon Catheter, Type N3.0-21-20-P-NS-MAC1, William Cook Europe A/S, Bjaeverskov, Denmark) was a kind gift from Professor Michael Marber, Rayne Institute, St. Thomas' Hospital, London. His group had used this catheter to deliver cationic liposomes into rabbit coronary arteries (Wright et al. 1998). The catheter was advanced into the chosen coronary artery until the coronary artery was occluded. This was done because Donahue et al had suggested that in isolated cultured rabbit ventricular myocytes, mixing of adenoviral vector with either red blood cells or radio-opaque contract material could reduce the efficiency of adenoviral mediated gene transfer (Donahue et al. 1997). Coronary occlusion by the catheter was confirmed by injection of radio-opaque contrast but this contrast was flushed from the coronary artery with 0.9% sodium chloride before injection of the adenoviral vector. In the original paper by Barr et al the concentration of virus used was of the order of 10^9 to 10^{10} pfu/ml with a volume of 1ml injected over 60 seconds. We chose to inject virus of similar concentration and volume but

decided to inject the virus over 6-10 seconds. The reason for this variation was that in isolated whole hearts, which had been infected by recirculation of adenoviral vector, optimal combined flow rate down both coronary arteries had been found to be 30 ml/min (Donahue et al. 1997). We estimated that right coronary flow would be less than one third of total coronary flow.

The procedural outcome of these experiments is tabulated (Table 5.1). Surviving animals were sacrificed 5 days following virus introduction and a 4mm horizontal section cut from mid ventricle. These tissue samples were stained for beta-galactosidase expression using the method previously described (Chapter 2, Page 86, method 2). Histological sections of the stained tissue samples were cut from wax blocks and counter-stained with Haematoxylin and Eosin as previously described (Chapter 2, Page 88). On light microscopy, characteristic blue cytoplasmic staining identified cells expressing the reporter gene product, beta-galactosidase. Cardiomyocytes were identified morphologically by their cross-striations.

Results

Of the four rabbits that underwent selective left coronary artery catheterisation, only one survived. The other three animals died during the procedure. These animals died despite resuscitative attempts including external direct current shock. Examination of the histological sections from the one surviving rabbit revealed groups of cardiomyocytes staining positively for the foreign gene product beta-galactosidase. The positively staining cells were in a perivascular and epicardial distribution.

Of the five animals that underwent selective right coronary artery catheterisation, all survived the procedure. However, one animal died within 24 hours of the procedure. Reassuringly, post-mortem examination of this animal revealed no evidence of respiratory tract inflammation as had been found in animals injected with contaminated virus in Chapter four. On examination of histological sections taken from the four surviving animals, cells staining positively for the foreign gene product beta-galactosidase were found in the epicardial, endocardial and trabeculated areas of the right ventricle. The left ventricular free wall served as a non-infused control when staining for the reporter gene product. In animals infused via the right coronary artery, no positively staining cells were detected in the distribution of the non-infused left coronary artery. The right coronary artery supplies blood to the right ventricular free wall and a portion of the posterior septum (Plate 5.1.).

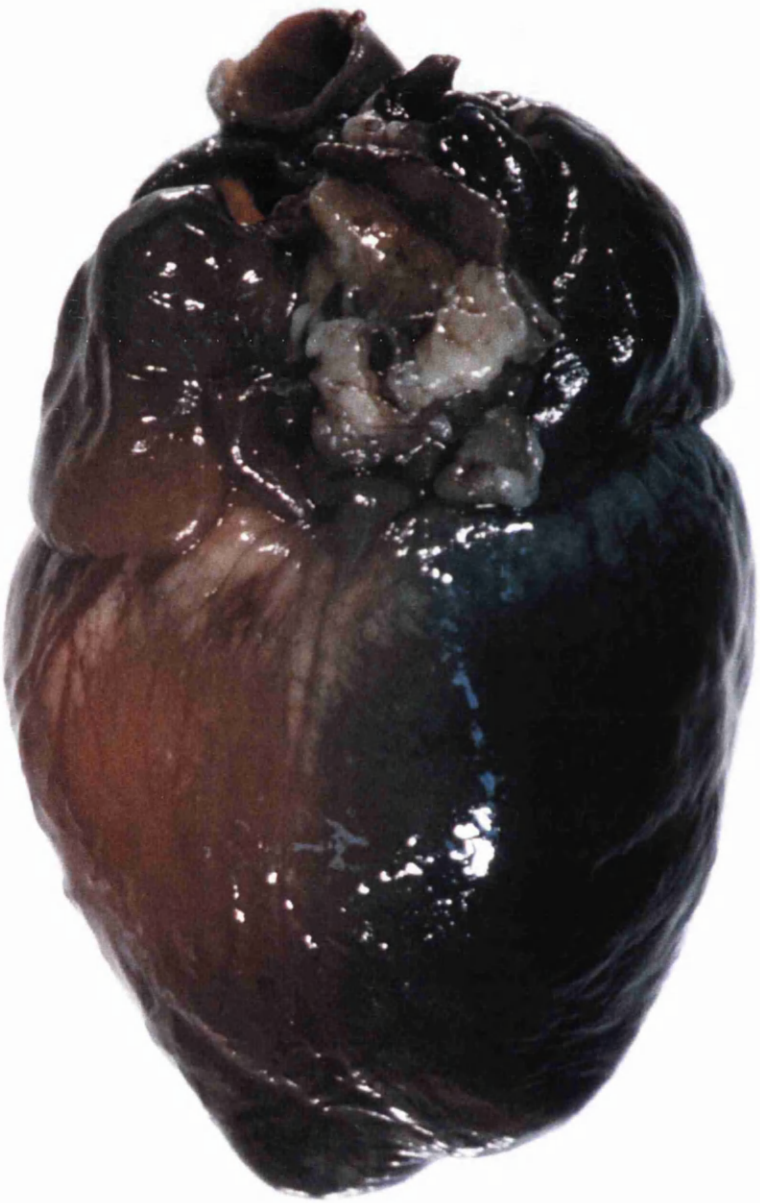


Plate 5.1. An example of a rabbit heart stained for beta-galactosidase expression (blue) 5 days following selective coronary injection of an adenoviral vector encoding beta-galactosidase. The heart is viewed from behind and the demarcation separating the left and right coronary territories is demonstrated.

Table 5-1 Procedural results of Selective Coronary Injection Experiments

Rabbit No	Weight (Kg)	Coronary Artery Injected	Day Sacrificed	Vector Concentration ($x10^9$ pfu/ml)	Volume Injected (ml)	Histology with X-gal stain
1613	3.4	Left	Died on table	0	0	None
1609	2.7	Left	5	13	0.3	Positive
1626	2.1	Left	Died on table	0	0	None
1637	2.3	Left	Died on table	0	0	None
1641	2.3	Right	1	4	2	None
1640	2.4	Right	5	4	1	Positive
1653	2.5	Right	5	4	0.9	Positive
1659	2.6	Right	5	2.5	1	Positive
1661	2.4	Right	5	5	1	Positive

Discussion

The long-term aim of the group was to deliver adenoviral vector encoding skeletal troponin C into the left coronary circulation with the intention of causing foreign gene expression in a large proportion of the left ventricular cardiac myocytes. If this method were successful, infected hearts would be suitable for physiological investigation using working heart methods. However, three of the four animals that underwent selective left coronary artery cannulation died quickly following introduction of the catheter. ECG monitoring was not performed during these procedures but the likely mechanism of death was ischaemia-induced arrhythmia. After injection of radio-opaque contrast material down a coronary artery, the artery typically remained radio-opaque until

either injection of physiological saline or withdrawal of the catheter. This would suggest that the selective coronary catheter completely occluded the coronary artery and that blood could not flow from the aorta into the cannulated coronary artery.

In the right coronary injection group the distribution of positively staining cells was curious. Although positively staining cells were found in the endocardial and epicardial regions of the right ventricular free wall, the middle region was devoid of positively staining cells (Plate 5.2.). However, there were some sections where the free right ventricular wall had been cut at an angle. In some of these sections positively staining cells were found in the middle region, but only at the cut edge (Plate 5.3.). This unusual appearance was consistent with non-penetrance of the X-gal stain beyond the surface layers of each tissue block.

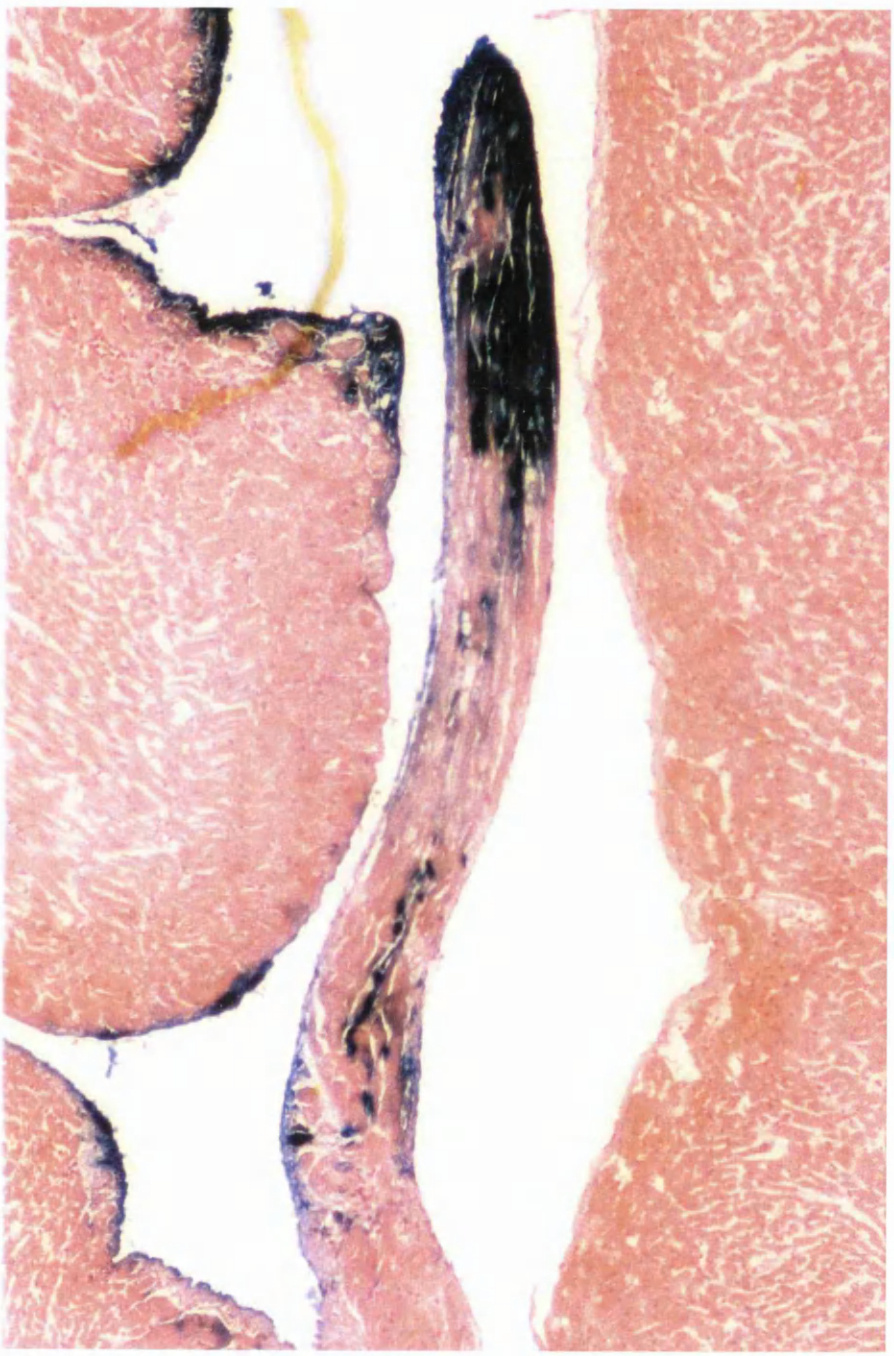


Plate 5.2. A histological section (magnification X10) of right ventricle taken from a rabbit 5 days following introduction of adenoviral vector encoding beta-galactosidase. Blue staining indicative of beta-galactosidase expression was detected in a trabecula and in the endocardial region of the right ventricular free wall.

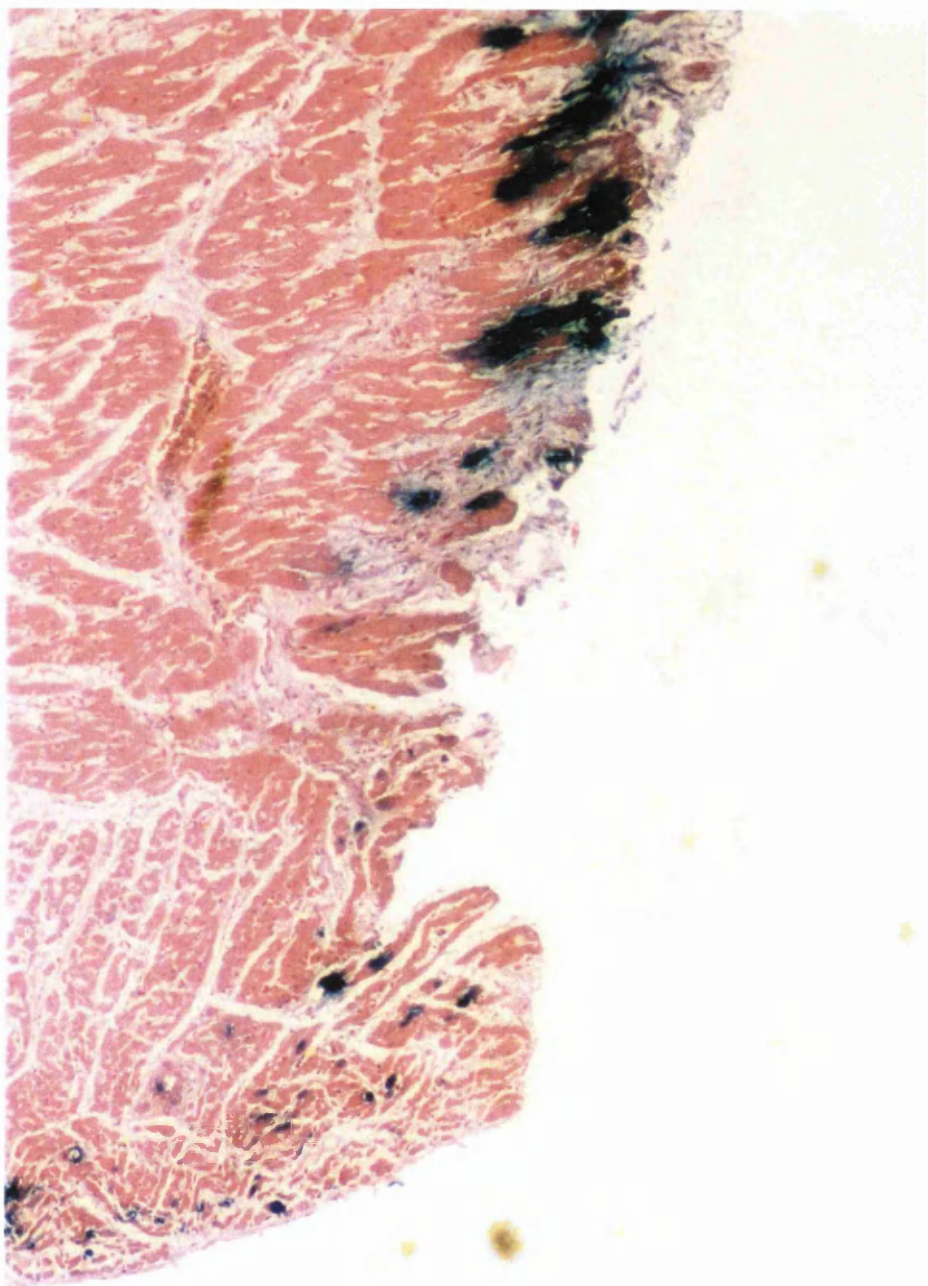


Plate 5.3. A histological section (magnification X25) of the cut edge of right ventricle taken from a rabbit 5 days following introduction of adenoviral vector encoding beta-galactosidase. Blue staining indicative of beta-galactosidase expression was detected at the cut edge but not throughout the full thickness of the section, suggesting non-penetrance of the staining solutions.

Conclusions

These experiments indicated that intra-coronary injection of adenoviral vector could not only cross the endothelial barrier but that it was possible to cause foreign gene expression in rabbit cardiomyocytes by this route. Although the method of staining used appeared specific for beta-galactosidase expression, there remained a concern about the sensitivity of the staining method used. In addition sham operated controls, and controls injected with vehicle or empty virus were not included during this development stage and the possibility of false positive staining, due to the procedure or virus in itself, was not excluded. The number of animals in these preliminary experiments was small. Nevertheless, temporary occlusion of the left coronary artery was poorly tolerated. The majority of the left coronary injection group died very quickly after selective coronary cannulation. Right coronary cannulations seemed better tolerated.

Selective right coronary artery injection became the preferred route for further development. The mass of the right ventricle is less than that of the left and it was anticipated that in a dose finding study, the total amount of virus required would be less if the right coronary artery route were used. Given the time-consuming nature of adenoviral vector propagation, this was an important practical consideration. There was local expertise in characterising the contractile physiology of rabbit

isolated skinned ventricular trabeculae. The methods used were suitable for determining the calcium sensitivity of infected and uninfected ventricular trabeculae over a range of pH. Ventricular trabeculae of suitable size had previously been found to be more numerous in the right ventricle than in the left (Denvir et al. 1996). The observation that right ventricular trabeculae had displayed foreign gene expression following introduction of adenoviral vector into the right coronary artery circulation made this the route of choice for further development.

CHAPTER 6

Comparison of Selective Coronary Injection and Aortic Cross-clamp Methods of Vector Delivery.

Introduction

The aim of this work was to determine the best practicable method of producing rabbit myocardial tissue suitable for investigation of contractile physiology. Within the group, attempts to produce an adenoviral vector which co-expressed skeletal troponin C and green fluorescent protein were unsuccessful. Such a vector would have had an application in selecting isolated single cardiac myocytes expressing foreign gene product. Other physiological methods available included the working heart preparation and measurement of isometric contraction in isolated ventricular trabeculae. To employ the working heart preparation would require a large proportion of the left ventricular cardiac myocytes to express the foreign gene of interest. This was unattractive given the practical difficulties encountered with both the Swan-Ganz and the selective left coronary cannulation methods. To address the question of whether adenoviral vector mediated expression of skeletal troponin C in rabbit myocardium can protect against the calcium desensitising effects of acidosis, isolated right ventricular trabeculae seemed the most attractive preparation. Efficient infection of right ventricular trabeculae would require introduction of vector into the right coronary circulation.

From the preliminary selective coronary injection experiments it was concluded that delivery of adenoviral vector into the coronary circulation could result in foreign gene expression in cardiac myocytes and non-myocytes. It was known from these preliminary experiments that infection of cells within right ventricular trabeculae was possible. However, the

concentration of virus required to infect a large proportion of cardiac myocytes had not been determined. To determine the effect of vector concentration on efficiency of infection, it was decided to perform further selective right coronary artery injections comparing vector concentrations of ~10^9 pfu/ml with ~10^{10} pfu/ml. The proportion of cardiac myocytes expressing foreign gene product would be determined by quantitative analysis of histological sections.

Whilst these experiments were being undertaken, discussion with Dr R Hajjar (Boston, Mass) led to consideration of an alternative approach. Dr Hajjar's group had been delivering replication defective adenoviral vectors encoding a nuclear-localising beta-galactosidase into the rat coronary circulation by a method analogous to the Swan-Ganz method. At open thoracotomy a cannula was introduced into the ascending aorta by direct puncture of the left ventricular apex and manipulation of the cannula through the aortic valve. The aorta above the catheter was clamped. However, by simultaneously clamping the pulmonary artery, left ventricular filling was reduced. When the virus was injected into the ascending aorta the only means of escape was down the coronary circulation. The transduction efficiency of this method as applied to the rabbit was unknown. This method had two theoretical advantages over the Swan-Ganz method. Firstly, the volume of the dead space in the ascending aorta could be minimised and the potential for mixing of vector with red blood cells limited. Secondly, by reducing left ventricular filling, the pressure within the left ventricular cavity could be maintained at a low

level favouring continued coronary flow. It was decided to compare the selective coronary injection method with this promising new method.

Donahue et al reported acceleration of widespread adenoviral gene transfer to isolated rabbit hearts by prior coronary perfusion with Tyrodes solution containing a low calcium content alone and more efficiently with the addition of serotonin (Donahue et al. 1998). They suggested that with procedures to increase vascular permeability, a single pass of adenoviral vector through the coronary circulation could be sufficient for transduction of a substantial proportion of the whole myocardium. It was decided to include a group of animals where 5-HT was pre-injected down the right coronary circulation before low dose adenoviral vector.

Methods

Adenoviral Vectors

A replication defective adenoviral vector encoding a beta-galactosidase reporter-gene under the control of the cytomegalovirus promoter had been constructed using a commercially available kit (Microbix Biosystems Inc, Toronto, Canada) and was the kind gift of Prof. Ron Hays, St Andrews University. A replication defective adenoviral vector

(pAdRSVβgal) encoding a *nuclear localising* beta-galactosidase gene under the control of the Rous Sarcoma Virus promoter was a kind gift from Roger Hajjar, Harvard Medical School, Boston (Dong, Schulick, DeYoung, & Dichek 1996). A replication defective adenoviral vector encoding skeletal troponin C with a haemagglutinin tag was constructed by Dr Obeid Khan (Khan 1999) with pE1A (Microbix) employing a previously described method (Hitt et al. 1995).

Histochemical Staining

Five days following vector introduction, the animals were sacrificed by intravenous injection of Euthetal and the hearts stained by Coronary Infusion as previously described (Chapter 2, Page 90). The composition of the staining solutions was as before but the solutions were infused down the coronary circulation to ensure that the full thickness of the myocardium was exposed to each solution. A 4mm thick horizontal section was cut from the mid ventricle and (with the exception of the low-dose selective coronary injection group) a separate tissue block cut from the trabeculae rich basal right ventricle. Tissue blocks were encased in wax from which standard 6-micron histological sections were cut and counter stained with Haematoxylin and Eosin as before (Chapter 2, Page 91).

Quantitative Analysis

Using light microscopy (Model DM1L, Leica, Wetzlar, Germany), H&E stained histological sections were examined for evidence of foreign gene expression, cell death and inflammatory infiltrate. The proportion of myocardium with detectable foreign gene expression was determined by point counting. An England Finder Grid (Graticules Pyser-SGI Ltd, Tonbridge) was inverted and randomly placed over the histological slide. At 40X magnification both the histological section and the grid were in focus. At each intersection of the grid the underlying cell type was identified by its morphological features. Cardiomyocytes were identified by their cross striations. The number of cardiomyocytes staining positively for the foreign gene product was noted together with the total number of cardiomyocytes counted. Where a cell did not overlay the intersection, the cell to the immediate upper left was counted. This was performed separately for the right ventricular free wall, trabeculae rich basal right ventricle and left ventricular free wall. For each region, the count was performed on five randomly selected grid positions. On three separate tissue samples, an independent observer who was blinded to the results was able to reproduce a count of the total number of cardiac myocytes per section to within 7% of the original estimated.

Results

Four groups of animals received adenovirus vector encoding a beta-galactosidase gene. The groups were termed "High-dose aortic cross-clamp", "High-dose selective coronary injection", "Low-dose selective coronary injection" and finally, "Low-dose selective coronary serotonin pre-injection". Animals were sacrificed five days following viral introduction and their hearts removed for histo-chemical analysis to detect beta-galactosidase expression.

In animals that underwent selective right coronary cannulation and viral infusion, the left ventricular free wall served as a non-infused control. Animals infused with 1 ml of $1x10^{10}$ pfu/ml of adenoviral vector encoding a skeletal troponin C mutant under the control of a human cardiac actin promoter pHCAsTnCHA served as infused controls (n=4). None of the tissues from non-infused control or infused control animals stained positively for the reporter gene product beta-galactosidase.

In the high-dose aortic cross-clamp group (n=8) each animal received 1ml of vector at a concentration of $1x10^{10}$ pfu/ml. Only occasional positively staining cardiomyocytes were found in the epicardial region of both ventricles, amounting to less than 1% of ventricular cardiomyocytes (data not shown). None of the right ventricular trabeculae from this group stained positively for the reporter gene product.

In the low-dose right coronary injection group, each animal received 1ml of vector at a concentration of $5x10^9$ plaque forming units (pfu)/ml (n=6). On histological examination, beta-galactosidase reporter gene expression was localised morphologically to perivascular cardiac myocytes and non-myocytes in the right ventricles of 5 animals (Plate 6.1). In these animals 2% (range 1.1%-3.3%) of right ventricular (RV) cardiomyocytes stained positively for the reporter gene product. The heart of only one animal displayed clear areas of inflammatory infiltration with associated cardiomyocyte cell death. This phenomenon has been termed micro-infarction and has been reported in previous studies involving myocardial infusion of liposomes (Wright et al 1998).

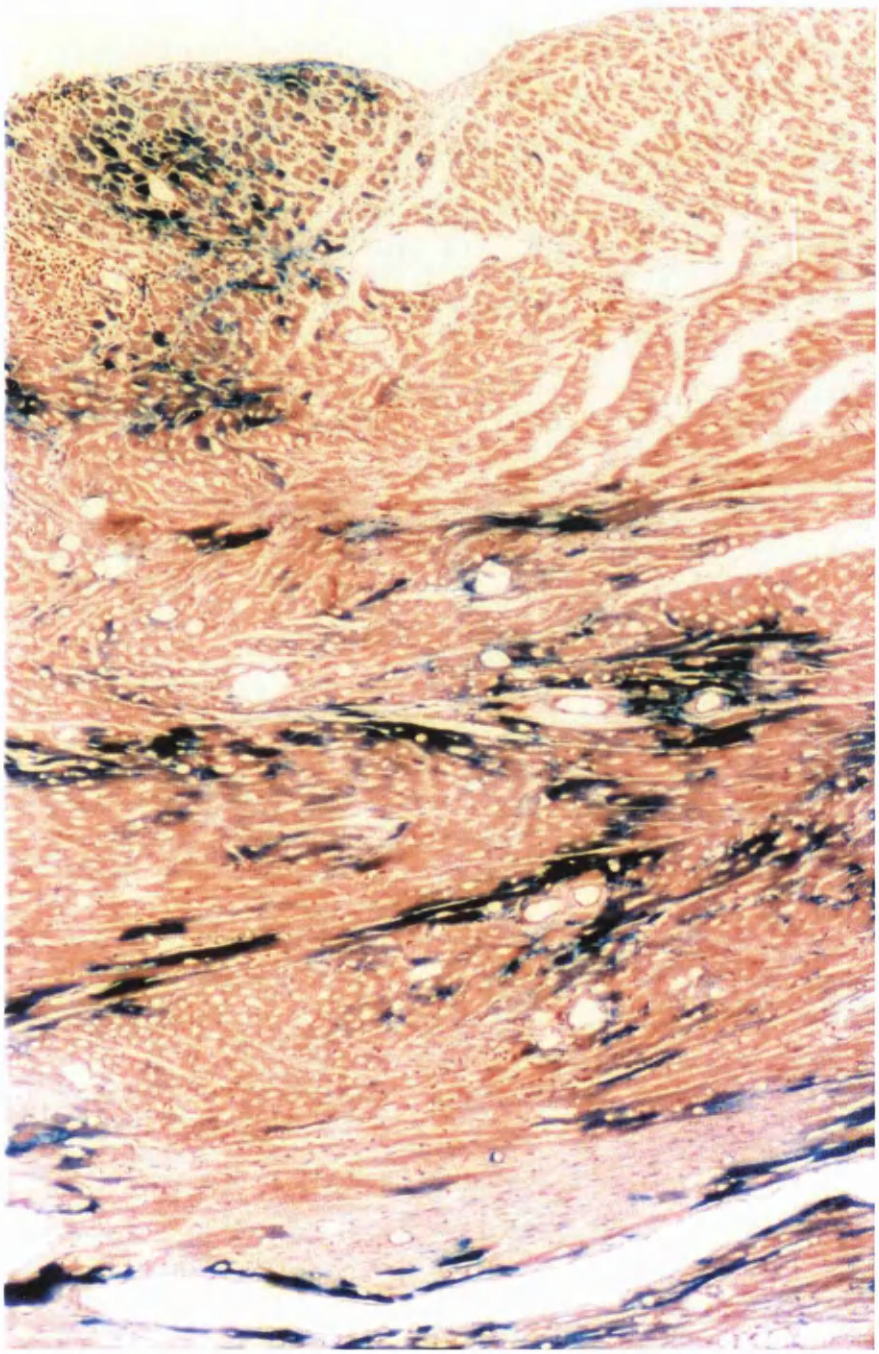


Plate 6.1. An example of an H&E stained section (X10 magnification) from the right ventricle of an animal 5 days after introduction of low-dose adenoviral vector encoding the beta-galactosidase gene. Cells staining positively for the reporter gene product appear blue.

In the high-dose right coronary injection group each animal received 1 ml of vector at a concentration of $1x10^{10}$ pfu/ml (n=8). On histological examination, reporter gene expression was detected in the hearts of 7 animals. In these animals 4.4% (range 0.2%-11%) of RV free wall cardiomyocytes and 10% (range 0.2%-31%) of RV trabecular cardiomyocytes stained positively for the reporter gene product beta-galactosidase (Plate 6.2, Plate 6.3). The level of reporter gene expression in the right ventricle was significantly greater in this group than in the high-dose aortic cross-clamp group, $p<0.05$. However, in this group, areas of inflammatory infiltration with associated cardiomyocyte cell death affected 32% (range 0%-64%) of the right ventricular free wall which differed significantly from levels obtained from the low-dose selective coronary injection group, $p<0.05$. In the group infused with 1 ml of $1x10^{10}$ pfu/ml of control virus encoding the troponin C mutant, myocardial infarction affected 24.2% (1.9%-64.4%) of the right ventricular free wall. Again, this differed significantly from levels obtained in the low-dose selective coronary injection group $p<0.05$.

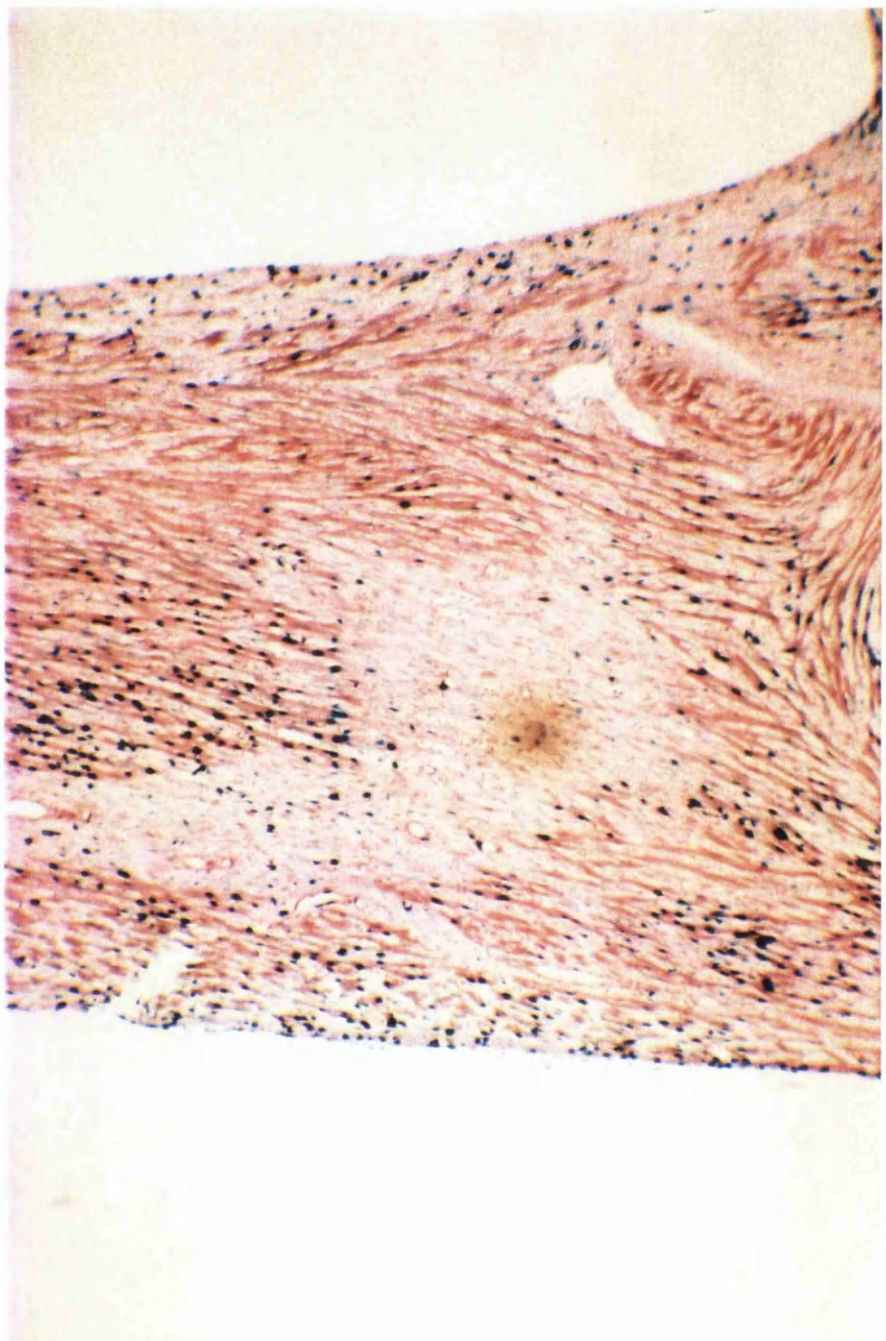


Plate 6.2. An example of an H&E stained section (X10 magnification) from the right ventricle of an animal 5 days after introduction of high-dose adenoviral vector encoding a nuclear-localising beta-galactosidase gene. Cells staining positively for the reporter gene product have blue nuclei. In this representative section there is an area of micro-infarction.

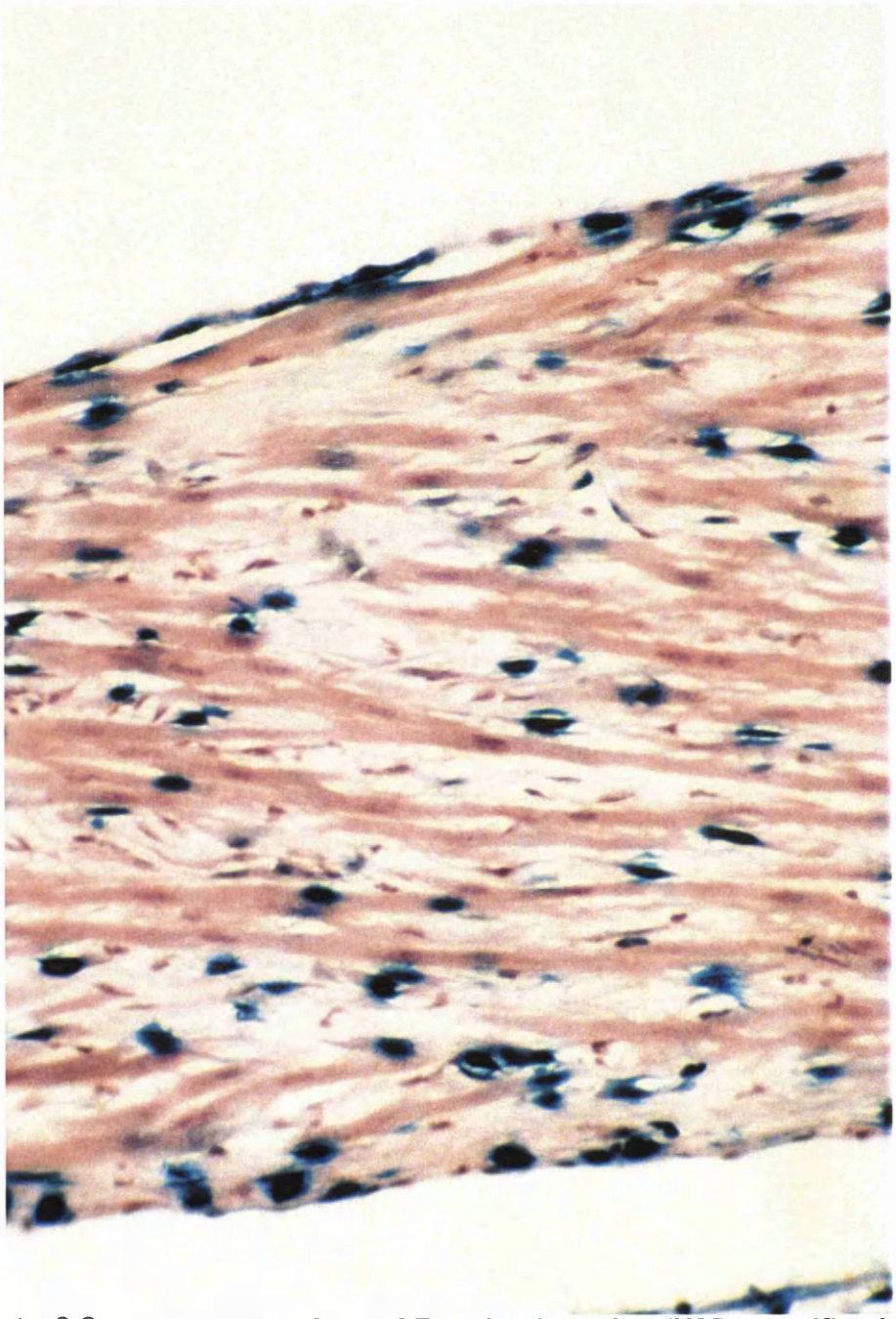


Plate 6.3. An example of an H&E stained section (X25 magnification) of a trabecula found in the right ventricle of an animal 5 days after introduction of high-dose adenoviral vector encoding a nuclear localising beta-galactosidase gene. Cells staining positively for the reporter gene product have blue nuclei.

In the final group, right coronary injections were performed using 1ml of vector at a concentration of $5x10^9$ plaque forming units (pfu)/ml (n=6). However, in this group, the radio-opaque contrast was flushed from the coronary circulation prior to virus injection with 1ml of 10^{-5}mol/L serotonin and not physiological saline as before. This group was termed "low-dose serotonin pre-injection group." On histological examination, beta-galactosidase reporter gene expression was localised morphologically to perivascular cardiac myocytes and non-myocytes in the right ventricles of 5 animals. In these animals 4.5% (range 1.4–6.8%) of RV free wall cardiomyocytes stained positively for the reporter gene product, which differed significantly from that obtained for infused or non-infused controls ($p<0.05$). However, in this group, areas of inflammatory infiltration with associated cardiomyocyte cell death affected 54% (range 7%-79%) of the right ventricular free wall and this was significantly greater than the low-dose coronary injection group without serotonin pre-injection ($p<0.05$). In the rabbits that underwent serotonin pre-injection, blood was taken before and one day after vector introduction. The blood was tested using the only commercially available kit to detect the presence of cardiac troponin T (Cardiac Reader System, Roche Diagnostics Ltd, East Sussex). All pre-injection samples were negative for troponin T. However, every sample taken one day following vector introduction tested positive for cardiac troponin T, indicating that some myocardial damage had occurred within one day of vector introduction. The histological data from the selective coronary injection groups are summarised in figure 6.1 and figure 6.2.

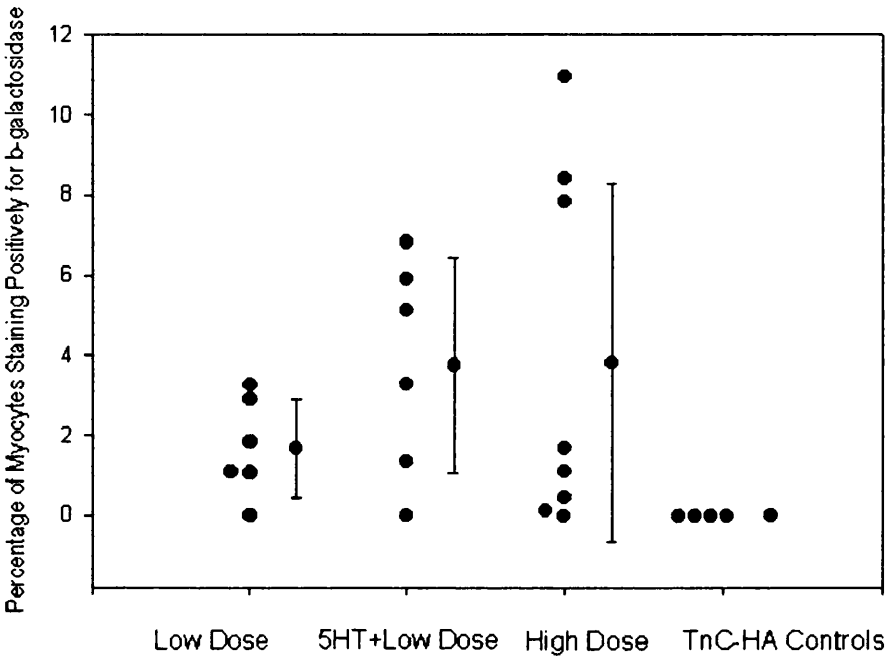


Figure 6.1. Percentage of right ventricular myocytes staining positively for the reporter gene beta-galactosidase. Point plot of all 4 coronary injection groups with mean and standard deviation for each group. Percentage of positive staining was Low Dose (n=6) 1.7+/- 1.2, 5HT+Low Dose (n=6) 3.7+/-2.6, High Dose (n=8) 3.8+/-4.4, TnC-HA Controls (n=4) 0.0+/-0.0

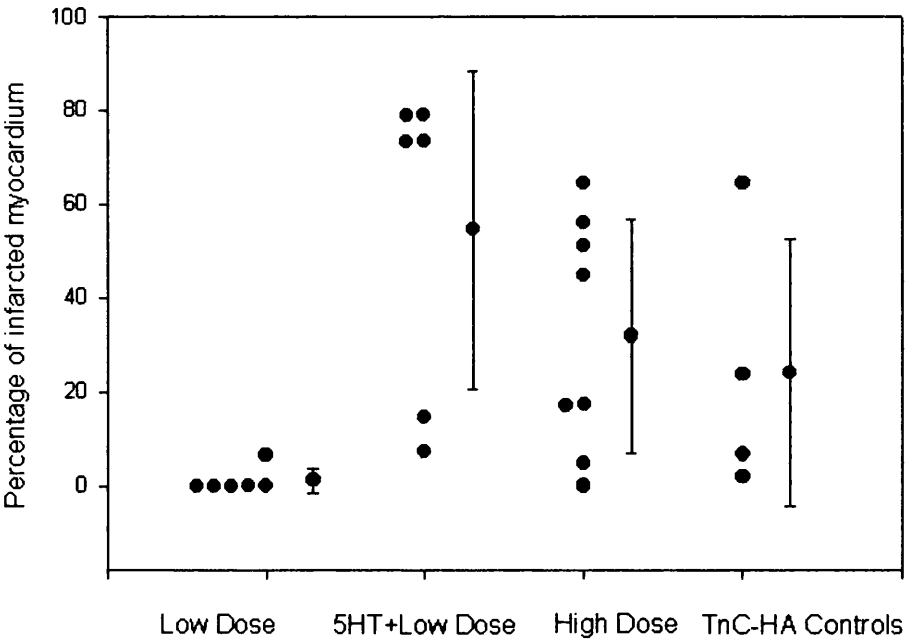


Figure 6.2. Percentage of micro-infarction in the right ventricle. Point plot of all 4 coronary injection groups with mean and standard deviation for each group. Percentage of micro-infarction was Low Dose (n=6) 1.1+/- 2.6, 5HT+Low Dose (n=6) 54.5+/-34.0, High Dose (n=8) 32.0+/-24.9, TnC-HA Controls (n=4) 24.2+/-28.4

Discussion

For the purpose of *in vivo* myocyte transduction of adult New Zealand white rabbit right ventricular trabeculae or right ventricular free wall, both the low and high-dose selective coronary injection method were found to be more efficient than the high-dose aortic cross-clamp method. In previously reported experiments involving cultured isolated cardiac myocytes the presence of red blood cells decreased transduction efficiency of adenoviral vector. Occluding the coronary artery with the catheter prior to viral injection reduced the risk of contaminating the viral

vector with red cells. In addition, the coronary artery was pre-injected with nominally calcium-free physiological saline before viral delivery, a process that in isolated Langendorff heart preparations has been reported to increase the proportion of viral load reaching cardiac myocytes. The mechanism of this effect was thought to be an increase in endothelial permeability to adenovirus (Donahue et al. 1998). The decision to inject the viral vector over 6-10 seconds was based on the coronary flow required for efficient gene transfer in the earlier reports of Langendorff adenovirus re-circulation experiments (Donahue et al. 1997).

In the high-dose selective coronary injection group, right ventricular trabeculae were found in which up to one third of their cardiomyocytes stained positively for the reporter gene product. It is possible to isolate right ventricular trabeculae suitable for studies in isometric tension measurement (Denvir, MacFarlane, Miller, & Cobbe 1996). One of the principal aims of the study was to investigate the calcium sensitivity of myocardial tissue that expressed skeletal troponin C following adenoviral mediated gene transfer. Permeabilised trabeculae can be used to investigate the effects of components of ischaemia and in particular, acidosis. Although the aortic cross-clamp method had failed to produce adequate efficiency of gene transfer to allow investigation of a whole heart preparation, it was hoped that important questions could still be answered by utilising isolated trabeculae.

Inflammation

In immune competent animals, inflammatory reactions associated with tissue transduction with first generation adenovirus vectors have been reported previously (Gilgenkrantz et al. 1995; Knowles et al. 1995; Li et al. 1995). In the low-dose selective coronary injection group there was little evidence of significant myocardial damage in all but one of the animals. All hearts were examined 5 days following viral introduction and this may be too early for a significant antibody mediated immune response. Nevertheless, these results were achieved without the use of an immunosuppressant such as methylprednisolone which has been used to decrease inflammatory reactions in studies of adenovirus delivery to myocardium (Shah et al. 2000).

Significantly more myocardial damage was seen in both the high-dose group and in the low-dose serotonin pre-injection groups (Student t test $p<0.05$, Mann-Whitney Rank Sum Test $p<0.05$ respectively). In both these groups significant micro-infarction was observed and could have been the result of a cell-mediated response or a prompt humoral response. The high proportion of infarcted myocardium found in the serotonin pre-injection group could be as a consequence of either a direct effect of serotonin on the myocardium or an indirect effect by substantially increasing the proportion of viral vector reaching the cardiac myocytes. The data from these experiments does not help distinguish between the two possibilities. The possibility remains that with modified adenovirus vector, serotonin may facilitate vascular permeability without increasing

micro-infarction. Further work is required to increase efficiency of delivery through the endothelial barrier and determine optimal dose requirements. It is likely that work performed in optimising vascular permeability to adenoviral vectors would be applicable to modified adenoviruses (Kochanek et al. 1996) and potentially to adeno-associated virus (Svensson et al. 1999).

Specificity of Beta-galactosidase Staining

In rabbit hearts infused with cationic liposomes, non transduced areas of micro-infarction were reported as staining positively using a method thought specific for beta-galactosidase activity (Wright et al. 1998). We found our staining method to be specific for nuclear-localising beta-galactosidase. In a group of rabbits with areas of micro-infarction following the introduction of high dose adenoviral vector encoding a skeletal troponin C mutant pHCAsTnC-HA, no positively staining cells were detected. In addition, the use of a nuclear-localising beta-galactosidase reporter gene simplified the morphological identification of striated cardiomyocytes. Why did some animals which had been exposed to a vector encoding a beta-galactosidase reporter gene fail to show any evidence of foreign gene expression? One possibility is that there was incomplete occlusion of the selected coronary artery by the catheter. This would allow blood to pass down the coronary artery, reducing the effect of nominally low-calcium saline injection on the endothelium and allow

vector to mix with red blood cells. Both of these factors would be expected to reduce transfection efficiency.

Conclusions

From these experiments it was concluded that the aortic cross-clamp method of vector delivery was inadequate for the efficient transduction of a sizable proportion of left ventricular cardiac myocytes. Indeed, the selective right coronary injection method was found to be a more efficient method of producing right ventricular trabeculae that expressed foreign gene product. The selective right coronary artery injection could produce right ventricular trabeculae with up to one third of their myocytes expressing foreign gene product. However, selective coronary injection of high concentration vector resulted in significantly more myocardial micro-infarction when compared with low concentration injections. Pre-injection of serotonin resulted in substantial myocardial damage even when low concentration vector was used. It is not clear from these data whether the mechanism by which damage was caused was by serotonin *per se* or by more vector reaching the myocytes through a more permeable endothelium. Further control groups should have included sham operated animals, and animals injected with vehicle alone or empty virus. The data from these negative control groups would have excluded the possibilities of both false positive staining and of a procedural cause for the micro-infarction seen. In addition two control group of animals pre-injected with serotonin and then injected with either vehicle alone or empty virus

should have been included. Data from these control groups would have helped clarify the mechanism by which serotonin resulted in substantial myocardial damage even when low concentration of vector was used. The lack of these important control groups seriously undermined the conclusions which could be reached from the data presented.

CHAPTER 7

Physiological Investigation of Right Ventricular Trabeculae.

Introduction

Selective right coronary injection of 1 ml of adenoviral vector at a concentration of $1x10^{10}$ pfu/ml had been found to result in expression of foreign gene product in up to one third of myocytes within the trabeculated area of the basal right ventricle. When the same volume and concentration of adenoviral vector was administered by the aortic cross-clamp method, only small numbers of left ventricular myocytes stained positively for foreign gene product (Chapter 6). The original hypothesis was that when compared to controls, adenoviral mediated expression of skeletal troponin C within rabbit myocardium would render the myocardium relatively resistant to the calcium desensitising effects of acidosis, a component of ischaemia. For the purposes of testing the original hypothesis the selective coronary injection method showed promise. It had been a pre-stated aim of the group to develop an adenoviral vector that would co-express both skeletal troponin C and Green Fluorescent Protein (GFP). If co-expression of GFP could be achieved then infected individual trabeculae could be distinguished from uninfected trabeculae by their visual appearance and isolated for physiological investigation. Using the selective right coronary injection method of adenoviral vector delivery, the proportion of positive staining cells within individual trabeculae was variable and the ability to pre-select only those trabeculae demonstrating foreign gene expression was

important. However, attempts at producing a suitable co-expressing vector had been unsuccessful.

CMV-sTnC-Δ9-HA Ad5 was a vector produced by Obeid Khan (Khan 1999). It was an adenoviral vector encoding skeletal troponin C. However, the sequence encoding the C-terminal nine amino acids had been removed and replaced with a 12 amino-acid sequence. This new sequence encoded 3 linker amino acids attached to a 9 amino-acid haemagglutinin antigen motif. Monoclonal antibodies with high affinity to the haemagglutinin antigen were commercially available. This vector had been constructed with the original intention that it be used in conjunction with confocal microscopy to determine the intracellular location of foreign gene product, and in particular, the incorporation of foreign gene product into the sarcomere. CMV-sTnC-Δ9-HA Ad5 was used by Dr Khan to infect NIH3T3 mouse fibroblasts cells in culture. A western blot for the presence of HA tag was positive in fibroblast cells infected with CMV-sTnC-Δ9-HA Ad5 but negative for cells infected with HCA-sTnC-Δ9-HA Ad5 and in uninfected cells. Both vectors were used to infect rat neonatal cardiocytes. Immunostaining for the HA product was positive in CMV-sTnC-Δ9-HA Ad5 infected cardiocytes and to a lesser extent HCA-sTnC-Δ9-HA Ad5 infected cardiocytes, but was negative in negative control groups. Dr Khan concluded that the vectors could cause foreign gene expression and that the HCA-sTnC-Δ9-HA Ad5 vector demonstrated characteristics of tissue specific expression. However, from these experiments it was not known if the HA tagged product integrated into the

sarcomere of infected myocytes. The C-terminal 9 amino-acids had been replaced by a 12 amino-acid sequence including the 9 amino-acid sequence for the HA tag. The C-terminal of TnC sits close to and interacts with the other troponin sub-units and the possibility remained that the lengthened HA tagged mutant may not integrate into the sarcomere. In retrospect, confirmation of integration of sTnC-HA into the sarcomere in cell culture should have been sought prior to proceeding to *in vivo* functional experiments.

Experimental Design

Adult New Zealand White rabbits underwent injection of 1ml of $1x10^{10}$ pfu/ml of adenoviral vector by selective right coronary injection as previously described (Chapter 6). One group of animals received vector encoding nuclear localising beta-galactosidase and a second group received the vector CMV-sTnC-Δ9-HA Ad5. Animals were sacrificed five days following vector introduction, and their hearts removed. The hearts were immediately placed in Modified Ringer's Solution containing 30mM BDM. The methods used to isolate, permeabilise and determine the calcium sensitivity of skinned fibres have been described in detail (Chapter 2, Page 97). Importantly, at the time that the trabeculae were isolated, it was not known what proportion of cardiac myocytes within each trabecula expressed foreign gene product. Following physiological investigation, trabeculae were taken for histochemical or immuno-

histochemical determination of the proportion of myocytes expressing foreign gene product. It was anticipated that from the two groups of animals, three groups of data would be available: trabeculae with a high proportion of cells expressing skeletal troponin C, trabeculae with a high proportion of cells expressing nuclear localising beta-galactosidase and trabeculae with no (or low) detectable foreign gene expression. The beta-galactosidase group was included as previous reports had indicated that adenoviral vectors per se could alter contractile characteristics (Novotny, Gustafson, Kvapil, & Ransnas 1994). Control groups including sham operated animals, animals injected with vehicle alone and animals injected with empty virus should have been included.

Immunohistochemistry

The detailed methodology for immuno-histochemical staining of rabbit myocardium expressing skeletal troponin C with the haemagglutinin antigen motif has been described (Chapter 2, Page 88). To determine an appropriate primary antibody dilution, histological sections were obtained from areas of rabbit myocardium that had undergone direct needle injection of adenoviral vector. The base of the heart had been injected with the vector CMV-sTnC-Δ9-HA Ad5. The apex of the heart had been injected with the vector HCA-sTnC Ad5, an adenoviral vector encoding skeletal troponin C under the control of the human actin promoter (Khan 1999). Each injection was of 0.1ml of vector at concentration of 10^{10}

pfu/ml. The animal was sacrificed 5 days following viral introduction. The results are summarised in Table 7.1.

Table 7.1 Immunohistochemistry Results of Direct Needle Injection Experiments

	Primary Antibody 1:50	Primary Antibody 1:100	Primary Antibody 1:200	No Primary Antibody
CMV-sTnC-Δ9-HA Ad5	+++	++	-	-
HCA-sTnC Ad5	-	-	-	-
Un-injected control LV	-	-	-	-

"+" denotes positive staining for the haemagglutinin antigen motif.

Tissue that had been injected with the vector CMV-sTnC-Δ9-HA Ad5 five days previously stained strongly with a primary antibody dilution of 1:50 (Plate 7.1.). At this primary antibody dilution, there was no false positive staining of tissue exposed to HCA-sTnC Ad5 or uninfected myocardium. Thus, the method was thought specific for detection of haemagglutinin antigen motif expression in rabbit myocardium infected with adenoviral vector. This method would subsequently be employed to identify foreign gene product in permeabilised trabeculae after physiological investigation.

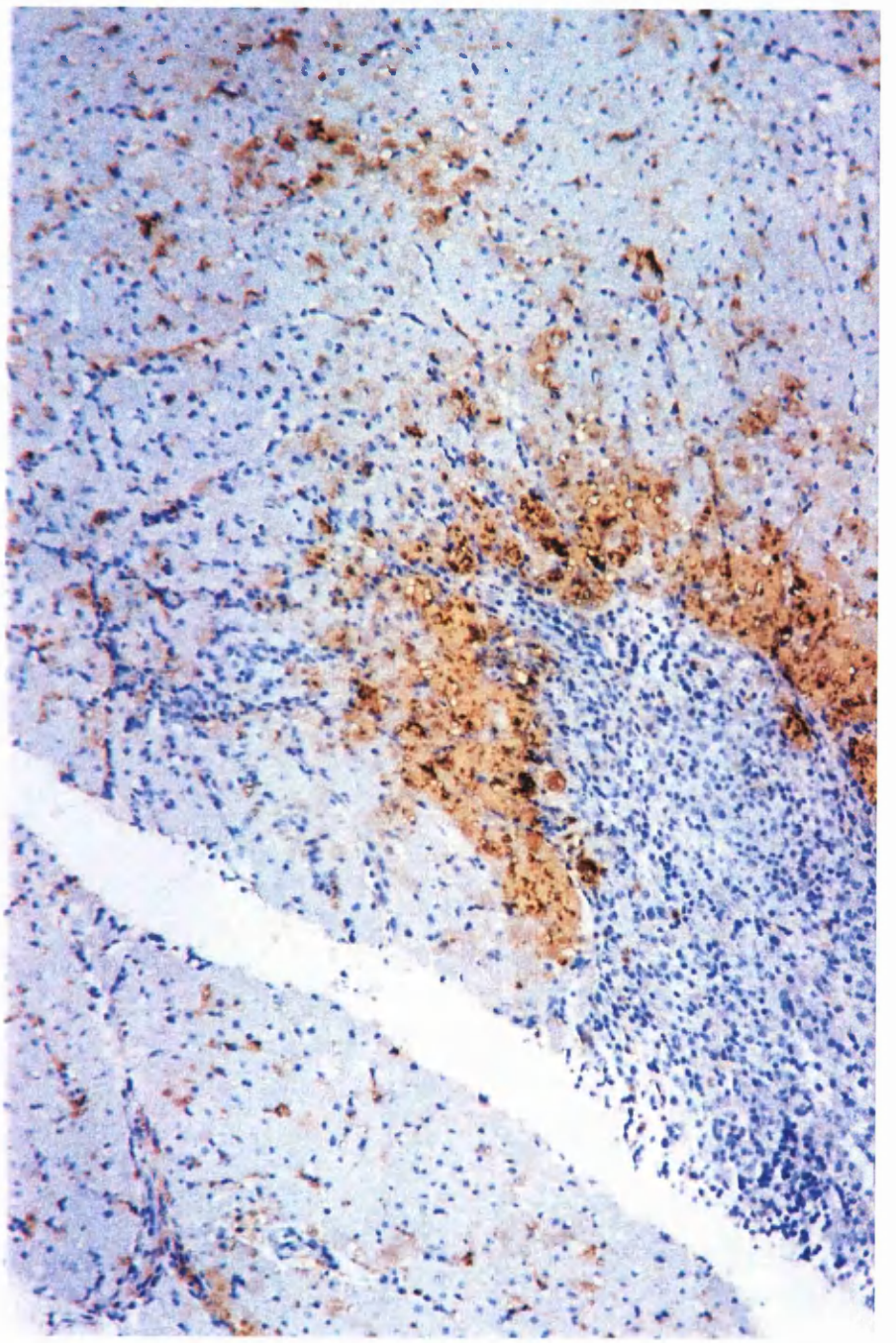


Plate 7.1. An example of a histological section of myocardium from a rabbit that underwent direct needle injection with the vector CMV-sTnC-Δ9-HA Ad5 five days previously. DAB staining was used and cells staining positively for the HA tagged protein appear brown.

Calcium Sensitivity of Permeabilised Trabeculae.

A total of eighteen trabeculae were isolated from ten adult New Zealand white rabbits. Six of the rabbits had been exposed to adenoviral vector encoding nuclear localising beta-galactosidase and nine trabeculae were tested from these animals. A further nine trabeculae were isolated from four rabbits which had been exposed to adenoviral vector CMV-sTnC-Δ9-HA Ad5. The methods used to determine the calcium sensitivity of these preparations at pH 6.5 and pH 7.0 have been described (Chapter 2, Page 92). These data were obtained without the operator knowing what proportion of myocytes in each isolated trabecula expressed foreign gene product. A macro running in the Mac application 'Igor Pro' (Written by Dr D Miller, using a Levenberg-Marquadt least squares algorithm) was used to generate a best-fit curve to the Hill equation for each preparation examined. From the curve thus derived, the calcium sensitivity for each preparation could be calculated and expressed in a standard manner as the free calcium concentration at which half maximal isometric tension was achieved (pCa_{50}). The results are summarised in Table 7.2

Table 7.2. Calcium Sensitivity of RV Trabeculae at pH 7.0 and 6.5

	Number of Trabeculae	Mean pCa_{50}	Standard Deviation	SEM
Beta-Gal pH7.0	9	5.505	0.121	0.0403
Beta-Gal pH6.5	9	4.929	0.154	0.0514
sTnC-HA pH 7.0	9	5.395	0.202	0.0673
sTnC-HA pH 6.5	9	4.952	0.213	0.0711

"Beta-Gal"- trabeculae from animals infused with vector encoding nuclear localising beta-galactosidase (n=9). "**sTnC-HA**"-trabeculae from animals infused with vector encoding sTnC with a HA tag motif (n=9). "**SEM**" – Standard Error of the Mean.

It was expected that within groups, there would be a significant reduction in calcium sensitivity at lower pH. However, it was not known if comparing between groups, trabeculae from animals infused with vector encoding the skeletal troponin C construct would be less sensitive to the effects of acidosis. An analysis was made on the data from all trabeculae studied before the results of histological and immuno-histochemical analysis was known. The pCa_{50} data from each group passed the Normality Test for normal distribution. Differences between groups were determined by employing a t-test. As expected there were significant reductions in calcium sensitivity between trabeculae studied at pH7.0 and pH6.5 (Beta-Gal pH7 v Beta-Gal pH6.5, $p<0.001$ and sTnC-HA pH7.0 v sTnC-HA

pH6.5, p=0.002). However, on comparing the pCa_{50} of trabeculae from Beta-Gal pH 6.5 with sTnC-HA pH 6.5 there was no significant difference (p=0.8). The mean pCa50 for these groups were 4.929 and 4.952 respectively. A power calculation was performed and with a difference in pCa_{50} of only 0.0229 between the groups, the power for two groups of 1000 rabbits would have been only 0.675 (assuming α of 0.05). It was anticipated that by selecting data from trabeculae with proven foreign gene expression, and excluding data from trabeculae with no histological evidence of foreign gene expression, the difference in pCa_{50} between groups would be greater and that a power calculation could be performed to estimate the number of experiments required. In a study of single cardiac myocytes from transgenic mice which expressed sTnC in their hearts, a significant difference was found between these and cells from normal hearts where n=11 in one group and n=7 in the other (Metzger et al. 1993).

Histology

Following physiological investigation, the permeabilised trabeculae were taken for histological examination. Trabeculae from animals infused with the vector encoding sTnC-HA were mounted in wax blocks and histological sections examined following immuno-histochemical staining using a high affinity rat antibody to the haemagglutinin epitope as described above. Trabeculae from animals infused with the vector encoding nuclear localising beta-galactosidase were immediately

immersed in the staining solutions for beta-galactosidase as had been used for the whole heart infusion (Chapter 2, Page 87). These trabeculae were stored overnight in wash solution at 4°C then mounted in wax blocks and sections cut. The results of the histological analysis were very disappointing: None of the trabeculae stained positively for their anticipated gene product. Using fresh antibody, further sections from animals infused with the vector encoding CMV-sTnC-Δ9-HA were carefully stained to detect the presence of foreign gene product. Again, no positive staining was observed.

Discussion

The method used to determine the calcium sensitivity of isolated rabbit trabeculae had been well characterised (Denvir, MacFarlane, Miller, & Cobbe 1996). However, the method requires complete disruption of the cellular and sarcoplasmic reticulum lipid membranes by Triton X-100. By effectively removing these membranes, the bath into which the preparation is immersed becomes an extension of the myocyte cytosol. By controlling the constituents of the bath solution, the cytosolic characteristics around the contractile matrices are controlled.

It was anticipated that up to 30% of myocytes in trabeculae from infused animals would express foreign gene product and that approximately one third of trabeculae would exhibit foreign gene expression in more than 10% of their myocytes. Since not all trabeculae were expected to display

foreign gene expression, methods of distinguishing infected trabeculae either before or after physiological investigation were required.

As expected, physiological experiments found clear reduction in calcium sensitivity of trabeculae at pH 6.5 when compared with trabeculae at pH 7.0. This was true for both the sTnC-HA and nuclear localising beta-galactosidase groups. The original hypothesis was that myocardial tissue expressing skeletal troponin C would be resistant to the calcium desensitising effects of intra-cellular acidosis. Although no significant difference was found between sTnC-HA pH 6.5 and B-gal pH 6.5, any real difference may have been attenuated by the presence of data from un-infected trabeculae within each group.

The most plausible explanation for none of the eighteen trabeculae staining positively for their expected gene product is that Triton X-100 completely disrupted all membranes. During physiological investigation, each trabecula underwent twenty-four bath changes at each pH. The two resting solutions used were of different ionic concentration and it is possible that both cytosolic and nuclear localising gene products would have been washed away. This potential problem had not been anticipated when the experimental protocol was decided upon. In retrospect *in vitro* experiments should have been undertaken to confirm that the sTnC HA-tagged mutant could be incorporated into the sarcomere of myocytes prior to proceeding to *in vivo* experiments. An alternative explanation for not detecting gene product was that this was a true negative. This was thought unlikely given the result of the original selective coronary injection

experiments (Chapter 6). In retrospect, the unused trabeculae from each animal should have been saved for later immunohistochemistry as this would have helped refute the possibility that none of the trabeculae expressed foreign gene product. The possibility of using *in-situ* hybridisation to detect viral DNA in histological slides of trabeculae was considered. However, adenoviral vector DNA remains epichromosomal and it too would be subject to "washout".

Conclusions

The results of the immuno-histochemical analysis of tissue taken following direct needle injection of rabbit myocardium demonstrate that the vector CMV-sTnC-Δ9-HA Ad5 can achieve detectable expression of its encoded gene product in rabbit myocardium. However, the presence of the gene product within the cell does not infer its incorporation into the sarcomere matrix and a physiological benefit remained to be demonstrated. In retrospect, *in vitro* experiments should have been undertaken to confirm that the sTnC HA-tagged mutant could be incorporated into the sarcomere of myocytes prior to proceeding to *in vivo* experiments.

It is well established that acidosis causes a fall in myocardial contractility (Poole-Wilson 1989). Irrespective of the vector infused, trabeculae from

both groups of animals demonstrated statistically significant reduction in calcium sensitivity in conditions of acidosis.

It had been expected that infected trabeculae from animals infused with CMV-sTnC-Δ9-HA Ad5 would be resistant to the calcium desensitising effects of acidosis when compared with trabeculae infected with vector encoding nuclear localising beta-galactosidase or compared with uninfected trabeculae. Control groups of sham operated animals, animals injected with empty virus and animals injected with vehicle alone should have been included. Had a the group injected with CMV-sTnC-Δ9-HA Ad5 been found to have significantly different physiological characteristic, the control groups would have been required to confirm that this was conferred by the presence of the gene product and not a consequence of the procedure or a non-specific effect of adenoviral vectors. Unfortunately, no histological confirmation of foreign gene expression could be demonstrated in any of the eighteen trabeculae that had undergone permeabilisation and physiological examination. From the original gene transfer results (Chapter 6) it would be possible but unlikely to have no detectable gene product following intra-coronary injection of high concentration adenoviral vector. An alternative explanation is that the Triton X-100 used to completely permeabilise all lipid membranes within the trabeculae allowed gene product to be washed away during the many bath changes required to determine calcium sensitivity.

When the calcium sensitivity of trabeculae at pH6.5 from animals infused with CMV-sTnC-Δ9-HA Ad5 was compared with that of trabeculae from

animals infused with vector encoding nuclear localising beta-galactosidase, only a small statistically insignificant difference was found. Without a method to identify the data from only infected trabeculae, a power calculation showed that the numbers needed to demonstrate a small difference would be impractical.

Selective coronary injection of adenoviral vector using the method described cannot guarantee that all RV trabeculae will express foreign gene product. An *in vivo* method of pre-selecting infected trabeculae for physiological investigation such as co-expression of Green Fluorescent Protein and the gene of interest would be advantageous.

Chapter 8

General Discussion

Introduction

The group of Hajjar were the first to demonstrate a change in heart function following the *in vivo* adenoviral mediated gene transfer of phospholamban (Hajjar et al. 1998). This was achieved by an aortic cross-clamp method in adult rats. Subsequent to this, the group of Koch reported successful *in vivo* delivery of an adenoviral vector encoding the potentially therapeutic gene for the beta-2 adrenergic receptor to rabbit myocardium using a modified aortic cross-clamp method (Maurice et al. 1999). Since then, Koch's group have reported global improvement in left ventricular contractility in response to isoproterenol in rabbits where an adenoviral vector encoding the gene for the beta-2 adrenergic receptor was delivered to rabbit myocardium by a selective coronary route (Shah et al. 2000). A direct comparison of the selective coronary injection with that of aortic cross-clamp method has not been reported before.

The group of Lawrence have employed isolated perfused rabbit hearts and cell culture methods to determine factors important to efficient adenoviral mediated gene transfer via the coronary circulation (Donahue et al. 1997; Donahue et al. 1998). Factors reported to reduce adenoviral mediated gene transfer include mixing of the virus with red blood cells or radio opaque contrast but not serum. Factors reported to improve efficiency of transfection in isolated perfused hearts include vector concentration, coronary flow rate and duration of exposure. However, a critical parameter to efficient adenoviral mediated gene transfer was

coronary circulation microvascular permeability. "Acceleration" of *ex vivo* gene transfer was reported in isolated perfused rabbit hearts where the Tyrodes solution had been modified and the calcium concentration reduced from 1mM to 50μM. The hypothesis was that low calcium perfusate would increase microvascular permeability and improve efficiency of gene transfer. Supportive of this hypothesis was that both serotonin and histamine, substances known to increase microvascular permeability, further improved transfection efficiencies. At optimal conditions, practically the whole heart was infected following only a short 2-minute exposure with a modest viral concentration of only 1.6×10^9 pfu/ml.

Replication defective adenoviral vectors can provoke an immune response which can result in damage to infected tissues (Gilgenkrantz et al. 1995; Kasseisler et al. 1994). In the isolated perfused heart experiments the heart could not be affected by the systemic immune system. In the selective coronary injection experiments by Koch's group an attempt was made to attenuate the potentially damaging effects of the immune system by administration of methylprednisolone. Nevertheless, the animals that received adenoviral vector exhibited unexplained increase in left ventricular end diastolic pressure and increases in resting heart rate. These changes were found in the group infused with vector encoding beta-galactosidase but not in the group infused only with saline. One potential mechanism for these findings would be immune mediated myocardial damage. Although a substantial increase in tissue gene product was reported, it is not clear if there was homogenous distribution

of infected myocytes or simply very high levels of gene product in perivascular myocytes. This is important, as heterogeneous expression of beta-2 adrenergic receptor would have the potential to cause heterogeneity of refractory periods within myocardium, and possibly a pro-arrhythmic effect.

Comparison of the Aortic Cross-clamp Method with Selective Coronary Injection

A direct comparison of the aortic-cross clamp method and the selective coronary method of adenoviral mediated gene transfer to myocardium had not been reported previously. In Chapter 6 the efficiency of the selective coronary injection method was compared to that of the aortic cross-clamp method and the selective coronary injection method was found to be superior. The viral concentration and dose reaching the coronary circulation was expected to be greater with the selective coronary method. However, the influence on coronary flow, microvascular permeability and left ventricular filling by simultaneous pulmonary occlusion in the Hajjar method were not known. The group of Koch have reported gene transfer to rabbit myocardium using a method which involves aortic cross-clamp but not pulmonary cross-clamp. This group used adenosine to temporarily slow or stop the heart. However, adenosine can cause coronary vasodilatation and it is not clear if the advantage of adenosine was one of only simple rate control.

The data in Chapter 6 demonstrate that low dose virus can result in transfection of cardiac myocytes within the distribution of the injected coronary artery without obvious myocardial damage five days following viral introduction. This is consistent with the previous report by Barr (Barr et al. 1994). However, when a higher concentration of vector was introduced by selective coronary injection, the proportion of myocytes expressing foreign gene was greater but significant myocardial cell death and inflammation was evident. This was detectable five days following viral introduction and this was thought early for a humoral mediated immune response. One possible explanation for early myocardial damage is vector induced cell death. In tissue culture experiments of isolated myocytes exposed to an adenoviral vector encoding for sarcoplasmic reticulum ATPase, viral titres of more than 4 pfu per cultured myocyte were associated with programmed cell death (O'Donnell et al 2001). The findings of substantial areas of myocardial damage in rabbits pre-injected with serotonin before low dose virus would be compatible with a harmful effect of serotonin *per se* or an increase in efficiency of viral delivery and a harmful effect of excessive numbers of viral particles reaching perivascular myocytes. However, the relevant controls with infusion of vehicle alone or infusion of empty virus were not performed.

Specificity of Histochemical and Immunohistochemical Methods

On staining the myocardium for detection of the expression of the reporter gene product beta-galactosidase the coronary perfusion method was found to be specific. No false positive staining was found in areas of microinfarction in animals infused with the vector encoding skeletal troponin C. However, these areas may not have been accessible to the X-gal solution if the inflammatory process resulted in capillary occlusion. Furthermore, the exact numbers of positively staining myocytes is subject to counting error because of indefinite myocyte borders and myocyte orientation. This was a particular concern when employing the non-nuclear localising beta-galactosidase. Nuclear localising beta-galactosidase did not demonstrate the same leakage into neighbouring cells but here there remained the possibility that the nuclei would not be in section and that cells expressing foreign gene product may be counted as not. This potential error would be expected to under-estimate the efficiency of gene transfer irrespective of the delivery method used. By counting only myocytes where the nucleus was visible, this potential problem was minimised.

The use of *in vivo* gene transfer of an HA tagged protein to rabbit myocardium had not been reported previously. The data from the direct needle injection experiment demonstrated that the high affinity monoclonal rat antibody could specifically detect haemagglutinin antigen

motif expression in rabbit myocardium exposed to replication defective adenoviral vector. This suggests that haemagglutinin antigen motif could have wider application and be used in the investigation of other genes of interest.

Use of Serum Troponin T to Detect Early Myocardial Damage

A sensitive method of detecting serum troponin T is available commercially (Roche, Welwyn Garden City). This is used clinically to detect minor myocardial damage in patients with acute coronary syndromes. We demonstrated detectable troponin T In the rabbits in which intra-coronary serotonin was delivered immediately prior to selective coronary injection of low concentration replication defective adenoviral vector. Troponin T, indicative of myocardial damage, was detected within 24 hours of infusion with adenoviral vector and it is unlikely that antibody mediated immune response would occur in this time course. Troponin T detection may have application in defining the time course of myocardial damage following gene transfer.

Physiology of Isolated Ventricular Trabeculae

The original hypothesis was that adenoviral mediated gene transfer of skeletal troponin C to rabbit myocardium would attenuate the calcium desensitising effects of acidosis, one of the components of ischaemia. If proven, the methods developed would have potential as a powerful investigative tool and would be the first step in developing skeletal troponin C as a potential therapy for ischaemic heart disease. It was known that adenoviral vectors had the potential to evoke an immune response and possibly cause myocardial damage. It was for this reason that the experimental design included tissue exposed to adenoviral vector encoding a physiologically inert reporter gene product (beta-galactosidase). By including this group and comparing physiological data with un-infected myocardium, there was the potential to elucidate the adverse effects of adenoviral mediated gene transfer on contractile physiology.

This was an ambitious goal as success was dependent on efficient gene transfer to a significant proportion of myocytes. Although Barr et al had reported this in 1994, their results proved difficult to reproduce. In addition, it was not known if skeletal troponin C would integrate into the sarcomere of infected cells. Integration of tropomyosin and troponin I into the sarcomere has been demonstrated in isolated myocytes following adenoviral mediated gene transfer (Michele, Albayya, & Metzger 1999).

Our original aim was to achieve foreign gene expression in a substantial proportion of myocytes in the left ventricle of adult rabbit hearts and to perform physiological investigations on isolated working hearts. Control of preload, afterload and heart rate is possible with the working heart preparation. True ischaemia can be induced by coronary artery ligation. However, using isolated trabeculae we were still able to test our original hypothesis in tissue from a model of gene transfer. The group of Hasenfuss have used the method of Donahue et al to infect isolated hearts by re-circulation of virus. Trabeculae isolated from these hearts remained viable for two days by which point foreign gene expression was detectable (Lehnart et al. 2000). This work provides an important tool for investigation of adenoviral-mediated alteration of contractile function. However, isolated trabeculae cannot remain viable indefinitely. Where gene products of interest take many days or weeks to integrate into the cell, this method may not be suitable. With adeno-associated viruses it may take several weeks before foreign gene expression is detectable (Wright et al. 2001). For this reason the method of Hasenfuss would not be attractive for investigation of adeno-associated virus mediated gene transfer.

Why foreign gene expression was not detected in any of the trabeculae that had undergone physiological investigation is uncertain. Selective coronary injection of adenoviral vector into the right coronary artery had been shown to result in foreign gene expression (Chapter 6). The histochemical and immunohistochemical methods employed to detect

foreign gene had been successful in whole hearts and histological sections respectively. The most likely explanation was that the detergent Triton X-100 completely dissolved all cell membranes including the nuclear membrane and that gene product was washed from the cell during frequent bath changes. There would be a clear advantage of being able to distinguish tissue which expresses foreign gene product from tissue that does not, prior to selection for physiological investigation. Co-expression of green fluorescent protein has such an application. This has been used to select single cells for unloaded velocity of contraction studies (Davia et al. 2001). If successfully applied in selecting infected trabeculae, data from isometric contraction would be obtainable.

Advances in Myocardial Gene Therapy

Vascular Endothelial Growth Factor has been the subject of great interest as a candidate for gene therapy in patients with ischaemic heart disease who are unsuitable for percutaneous coronary intervention or coronary artery bypass grafting. The group of Isner have reported promising results in patients in whom plasmid DNA encoding Vascular Endothelial Growth Factor under the control of the cytomegalovirus promoter was introduced to the myocardium by direct needle injection. This was performed on patients with inoperable ischaemic heart disease and severe symptoms. Preliminary results are that patient's symptoms are improved, as are myocardial perfusion images (Esakof et al. 1999; Kalka et al. 2000; Lathi

et al. 2001; Losordo et al. 1998; Symes et al. 1999; Vale et al. 1999a; Vale et al. 1999b; Vale et al. 2000; Vale et al. 2001). Vascular Endothelial Growth Factor is thought to act as a chemotactic agent and encourages the growth of new blood vessels towards the injection sites. Vascular endothelial Growth Factor has an advantage over many other gene therapy candidates in that only small numbers of myocardial cells need to express the gene product for the strategy to be effective.

The expression of sarcoplasmic reticulum ATPase is down regulated in the myocardial tissue of patients with heart failure and animal models of heart failure. In animal models the group of Hajjar have demonstrated that adenoviral vector mediated gene transfer can be used to up-regulate the expression of myocardial sarcoplasmic reticulum ATPase (Davia et al. 2001; del Monte et al. 2001; del Monte, Hajjar, & Harding 2001; del Monte et al. 1999). For this strategy to be effective in patients, there is a need to develop efficient methods of homogeneous gene transfer to the majority of myocytes within the heart.

Selection of Vector System

Adenoviral vectors have a large inset capacity and can easily accommodate insertion cassettes of 7Kb. In a "gutless" form inserts of 28Kb have been achieved (Clemens et al. 1996). They can be propagated to high titre and infect a wide variety of cells by attachment to Coxsackie adenovirus receptor and enter the cell by way of an integrin mediated process (Fechner et al. 1999). First generation adenoviral vectors were replication defective because they lacked the E1 region, essential for replication. In subsequent generations of vector other regions were deleted including E3. However, E3 products may be important in suppression of immune response (Efrat et al. 2001). Where transient expression in a small number of myocytes is required, adenoviral vectors may be useful. However, for the purposes of long-term gene transfer to large volumes of myocardium, these vectors are limited by transient expression and inflammatory damage. The transient expression is thought to be immune mediated. In athymic nude rats injected with an adenoviral vector encoding beta-galactosidase, foreign gene expression was detectable in these animals 120 days following virus introduction but not in similarly injected immunocompetent Sprague-Dawley rats (Quinones et al. 1996).

There is increasing interest in adeno-associated vectors. Although these vectors are difficult to propagate to high titre and have a smaller capacity for insertion cassettes, they can infect myocardial cells and cause foreign

gene expression without appreciable inflammatory response (Svensson et al. 1999; Wright, Wightman et al. 2001). Unlike adenoviral vectors where the encoded gene of interest remains episomal, adeno-associated vectors result in integration of the encoded gene of interest into the host genome. Adeno-associated viruses may have particular application where long-term foreign gene expression is desirable in a large proportion of myocardial cells.

Adenoviral Vector Production.

In the experiments reported here we used a traditional method of propagating and purifying replication defective adenoviral vector. To reduce the risk of contamination by replication competent virus, vector was propagated from single plaques and checked by restriction enzyme assay prior to use. However, one limitation of adenoviral vector is that it can never be guaranteed that the vector is completely free of replication competent vector. The most sensitive method of detecting replication competent contamination is by the supernatant rescue assay method (Dion, Fang, & Garver, Jr. 1996). Even here, the detection rate is 1 wild type replication competent virus in 10^9 of recombinant virus. The life cycle of adenoviral vector culminates in lysis of the host cell and spillage of many thousands of virons to neighbouring cells. With cell lysis occurring typically three days following viral exposure, it is possible that some of the myocardial damage seen in our delivery experiments at day 5 could have resulted from contamination by replication competent virus. However, if this was the case then the same effect would have been

expected in the low concentration selective coronary injection group and in the high concentration aortic cross-clamp group. The absence of intense inflammatory reaction in these groups would be against significant contamination by replication competent virus.

There has been the suggestion that at very high titre, adenoviral vector can precipitate to form visible crystalline aggregates. The highest concentration of vector used in the experiments reported here was of the order of 10^{10} pfu/ml. The vector had been purified with one round of caesium chloride gradient ultra centrifugation and although a 2μ filter was not used, at room temperature the vector was clear to the naked eye.

Following purification on a caesium chloride gradient, vector was desalted by dialysis and aliquots placed into Eppendorf tubes to be stored at –20°C. The buffer did not contain Glycerol and it is possible that the freezing process could have damaged some of the viral particles. The assay used to determine viral concentration was a traditional bioassay for plaques on agar covered HEK293 cells. In retrospect it would have been better to perform a bioassay and a light absorption assay to determine the number of defective particles contaminating each thawed aliquot. It is not known if the presence of defective non-replicating viral vector may have contributed to the inflammatory process seen in some animals.

Methods of Vector Delivery to Myocardium

There is interest in correcting the inherited conditions that predispose to life threatening ventricular arrhythmias. In the original report by Barr et al 1994, foreign gene expression was seen in a perivascular distribution following selective coronary injection of adenoviral vector. The results in Chapter 6 confirm this finding. Retrograde infusion of saline into the coronary sinus whilst infusing adenovirus into the coronary artery circulation has been used to improve efficiency of gene transfer in isolated rat hearts (Logeart et al. 2001). Interestingly, retrograde infusion of virus into the myocardial venous system has been employed to achieve homogeneous gene expression in the infused vascular territory (Boekstegers et al. 2000). One group have reported ultra-sound mediated destruction of albumin and adenoviral vector coated micro-bubbles to successfully direct intravenously injected vector to rat heart *in vivo* (Shohet et al. 2000).

Potential therapeutic genes where widespread transfection would be potentially advantageous include skeletal troponin C, sarcoplasmic reticulum ATPase and Heat Shock Protein. The rationales for the first two have been discussed. Heat shock protein may protect myocardium from reperfusion or thermal injury and may have an application as a prophylactic treatment in patients at risk of coronary artery occlusion (Brar et al. 1999; Gray, Amrani, & Yacoub 1999; Jayakumar et al. 2000; Jayakumar et al. 2001; Mestril et al. 1996; Okubo et al. 2001).

However, there may be methods to further refine selective coronary artery injection. From the work of Donahue et al it is important to prevent mixing of virus with red blood cells. The method of selective coronary injection used in Chapter 6 has been described in detail. On introducing the selective coronary catheter into the right coronary artery, radio-opaque contrast was injected to delineate the coronary artery. The artery was judged to be blocked by the catheter if the contrast material remained in the artery. However, it is possible that the vessel occlusion was incomplete in some animals. If the occlusion was incomplete then the microvascular permeabilising effect of pre-injecting (nominally calcium free) physiological saline would be reduced as would the efficiency of adenoviral vector mixed with red blood cells. This is one possible explanation for the variability found between animals in the proportion of myocardium transfected following apparently identical protocols. The selective coronary injection method could be modified to include a pressure transducer on a side arm connected to the coronary catheter. This could be used to ensure complete occlusion of the selected coronary artery and measure pressure. These measures may help achieve consistency in efficiency of transfection within groups.

The conditions required for optimal *in vivo* microvascular permeabilisation remain to be fully investigated. Solutions of low calcium content are effective but the additive effect of serotonin, histamine, VEGF and phosphodiesterase-5 inhibitors deserve further attention (Nagata et al. 2001). It is likely that measures that achieve efficient delivery of adenoviral vectors would be applicable to adeno-associated viruses.

It is possible to inject adenoviral vectors into the pericardial space. The superficial epicardial cells are transfected but homogenous or functionally significant gene transfer is yet to be demonstrated using this method (Fromes et al. 1999).

The results of the experiments in which a Swan-Ganz catheter was employed have been detailed in Chapter 4. The virus used in these experiments was subsequently found to be contaminated with replication competent virus. Once the vector quality issues were addressed, this method was not developed further. Unattractive aspects of this method included mixing of virus with red blood cells and contrast in the ascending aorta; non-occlusion of the pulmonary artery and high left ventricular cavity pressures resulting in a reduction in coronary flow. The group of Koch have used intra-ventricular injection of virus with aortic occlusion and achieved gene transfer of functional significance without pulmonary artery occlusion. This was achieved by pre-injection of adenosine to temporarily arrest the heart by blocking AV node conduction. The attraction of the Swan-Ganz method was that the arterial circulation of both ventricles would be exposed to vector without the risk of damaging either coronary artery. Perhaps with adenosine pre-injection the Swan-Ganz method could be revisited.

General Conclusion

This work has given me the opportunity to learn and become competent in a wide range of techniques to deliver vectors to myocardial tissue. I have gained an understanding of the different vector systems available and I have gained experience in amplification of adenoviral vectors. Despite amplification of single isolated plaques and testing of the resulting viral DNA by restriction enzyme analysis, the possibility of contamination by replication competent virus remained. In retrospect the supernatant rescue bioassay should have been utilised. I encountered problems with the specificity and sensitivity of the original histochemical method employed to detect beta-galactosidase expression in transfected myocardium. In response I employed a vector encoding a nuclear localising beta-galactosidase and modified the staining solutions to avoid false positive Prussian Blue staining. The sensitivity of the staining method was improved by retrograde infusion of staining solutions down the aorta and into the coronary circulation. I developed and optimised an immunohistochemical method of detecting the HA-tagged sTnC mutant following introduction into rabbit myocardium. In addition, I gained experience in using small isometric muscle preparations.

In the time since the original paper by Barr et al in 1994, to completion of the experiments presented here, no other group had published data confirming the original findings. Whilst developing the method of gene transfer to be employed, changes were made to the methods used in an

apparently unsystematic manner. In particular the Swan-Ganz method was abandoned. This decision was taken in response to the data by Donahue et al published in 1997.

The results of the coronary injection and aortic cross-clamp experiments were presented in oral and abstract form and were the first to compare the two methods in the rabbit. However, sham operated controls and controls injected only with vehicle were not performed. A control group injected with a adenoviral vector encoding a sTnC mutant was used and although it is reassuring that no false positive beta-galactosidase staining was found, empty virus would have been a better control here.

A serious flaw was to have proceeded to *in vivo* gene transfer of the sTnC HA-tagged mutant and physiological investigation of the exposed myocardial tissue without first confirming integration of the gene product into the myocyte sarcomere *in vitro.*

If I were to do it all again what would I do differently? I would not accept adenoviral vector for amplification without first performing quality assurance checks. In particular, I would screen for wild type contamination by the supernatant rescue bioassay method. In future, "clinical grade" vector is likely to be required which has been screened for a range of contaminants including wild type adenovirus, Hepatitis A virus, Herpes Simplex-1 virus, Murine Leukaemia virus, Porcine Parvovirus, Bovine Viral Diarrhoea virus, Cytomegalovirus and Minute Mouse Virus. In addition contamination by mycoplasma and bacteria endotoxins should

be excluded. There are now commercial organisations which provide such quality assurance services ((Microbix Biosystems Inc, Toronto, Canada). At each stage of development I would decide upon the experimental design, including appropriate positive and negative controls and perform the controls concurrently within the planned experiments. Finally, I would answer as many of the relevant questions using *in vitro* methods before proceeding to *in vivo* work.

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