Kewal K. Jain

# Applications of Biotechnology in Cardiovascular Therapeutics

Humana Press

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*This book is dedicated to*
*Prof. Dr. Friedrich S. Eckstein,*
*Chief of Cardiac Surgery, Heart Center,*
*University Hospital Basel, who performed*
*the life-saving coronary artery bypass open*
*heart surgery with smooth recovery to enable*
*me to write this book. My thanks are due to*
*PD Dr. Michael Zellweger of the Cardiology*
*Department for competently conducting*
*diagnostic investigations including coronary*
*angiography and arranging treatment.*
*Finally, Prof. Dr. Michael Tamm and*
*PD Dr. Werner Strobel of the Pulmonology*
*Department referred me for cardiac*
*investigations after I presented with*
*nonspecific dyspnea. Having visited some*
*of the best medical centers in the world,*
*I am happy to say that the treatment that*
*I received in Basel was optimal and second*
*to none. I learned a fair bit about modern*
*cardiology during my interaction with*
*physicians at the hospital.*

# Preface

This report puts together excerpts from the various writings by the author on the biotechnology topics as they relate to cardiovascular disease. Very appropriately the report was put together during the week that the author was recovering from open heart bypass surgery at the University Hospital, Basel, Switzerland. It is meant for physicians, surgeons, and scientists working on cardiovascular disorders. It will be useful for those working in life sciences and pharmaceutical industries, and some basics of cardiovascular diseases are included for nonmedical readers.

A major application of biotechnology is in therapeutic delivery to the cardiovascular system. Routes of drug delivery and applications to various diseases are described. Formulations for drug delivery to the cardiovascular system range from controlled release preparations to delivery of proteins and peptides. Various methods of improving systemic administration of drugs for cardiovascular disorders are described including the use of nanotechnology.

Cell-selective-targeted drug delivery has emerged as one of the most significant areas of biotechnology engineering research to optimize the therapeutic efficacy of a drug by strictly localizing its pharmacological activity to a pathophysiologically relevant tissue system. These concepts have been applied to targeted drug delivery to the cardiovascular system. Finally, devices for drug delivery to the cardiovascular system are described. A full chapter is devoted to drug-eluting stents used for treatment of restenosis following stenting of coronary arteries. This is one of the biggest segments of the cardiovascular drug delivery market with 15 companies involved in developing and producing stents.

Cell and gene therapies, including antisense and RNA interference, are described in full chapters as they are the most innovative methods of delivery of therapeutics. New cell-based therapeutic strategies are being developed in response to the shortcomings of available treatments for heart disease. Potential repair by cell grafting or mobilizing endogenous cells holds particular attraction in heart disease, where the meager capacity for cardiomyocyte proliferation likely contributes to the irreversibility of heart failure. Cell therapy approaches include attempts to reinitiate cardiomyocyte proliferation in the adult, conversion of fibroblasts to contractile myocytes,

conversion of bone marrow stem cells into cardiomyocytes, and transplantation of myocytes or other cells into injured myocardium.

Advances in molecular pathophysiology of cardiovascular diseases have brought gene therapy within the realm of possibility as a novel approach to treatment of these diseases. It is hoped that gene therapy will be less expensive and affordable because the techniques involved are simpler than those involved in cardiac bypass surgery, heart transplantation, and stent implantation. Gene therapy would be a more physiologic approach to deliver vasoprotective molecules to the site of vascular lesion. Gene therapy is not only a sophisticated method of drug delivery; it may also need drug delivery devices such as catheters for transfer of genes to various parts of the cardiovascular system.

Finally, a chapter on personalized cardiology is important for the era of personalized medicine. This concept is the best way of integrating new technologies in cardiology to select the best treatment for an individual patient.

The bibliography includes selected references from recent literature on this topic, which are appended to each chapter. The text is supplemented by 22 tables and 13 figures.

Kewal K. Jain, MD
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# About This Book

This book contains excerpts from various biotechnology books and reports authored by Prof. K. K. Jain that are relevant to cardiovascular disorders. The most important contributions of biotechnology are to cardiovascular drug delivery. Advances in cardiovascular surgery based on biomedical technology are beyond the scope of this report. This is the draft of the expanded and updated document to be issued as separate book.

# About the Author

Professor Kewal K. Jain is a neurologist/neurosurgeon by training and since his retirement from neurosurgery has been working in the biotechnology/biopharmaceuticals industry as a consultant at Jain PharmaBiotech. He received graduate training in both Europe and North America and has held academic positions in several other countries. He passed specialist examinations in neurosurgery in USA, Canada, and Australia. Currently, he is a Fellow of the Royal Australasian College of Surgeons and a Fellow of the Faculty of Pharmaceutical Medicine of the Royal College of Physicians of UK. Prof. Jain is the author of 425 publications including 18 books (2 as editor) and 49 special reports, which have covered important areas in neurosciences, biomedicine, biotechnology, cell/gene therapy, and biopharmaceuticals. In the 1970s, he developed a technique for sutureless microvascular anastomosis using lasers described in his *Handbook of Laser Neurosurgery* published by Charles C Thomas in 1984. His *Textbook of Gene Therapy* was translated into Chinese language in 2000. The *Textbook of Hyperbaric Medicine* (5th Ed 2009) has been a standard reference on the subject for the past two decades and contains a chapter on cardiovascular disorders.

Prof. Jain has edited *Drug Delivery Systems* (2008) and *Drug Delivery to the Central Nervous System* (2010), both published by Humana/Springer. His other recent books include *Handbook of Nanomedicine* (Springer/Humana 2008), *Textbook of Personalized Medicine* (Springer 2009), *Handbook of Biomarkers* (Springer 2010), and *Handbook of Neuroprotection* (Springer 2011).

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# Abbreviations

| | |
|---|---|
| ACE | Angiotensin-converting-enzyme |
| CAD | Coronary artery disease |
| CHD | Coronary heart disease |
| CHF | Congestive heart failure |
| CR | Controlled release |
| CVS | Cardiovascular system |
| DDS | Drug delivery system |
| DES | Drug-eluting stent |
| eNOS | Endothelial nitric oxide synthase |
| EPC | Endothelial progenitor cells |
| ESC | Embryonic stem cell |
| HDL | High-density lipoprotein |
| hESCs | Human embryonic stem cells |
| HSCs | Hematopoietic stem cells |
| IHD | Ischemic heart disease |
| LDL | Low-density lipoprotein |
| miRNA | microRNA |
| NIH | Nation Institutes of Health, USA |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| PAD | Peripheral arterial disease |
| PCR | Polymerase chain reaction |
| PEG | Poly(ethylene glycol) |
| PTCA | Percutaneous transluminal coronary angioplasty |

| | |
|---|---|
| RNAi | RNA interference |
| SC | Stem cell |
| siRNA | Short interfering RNAs |
| VEGF | Vascular endothelial growth factor |

# Chapter 1
# Cardiovascular Therapeutics

## Introduction

Drug therapy of the cardiovascular system is different from delivery to other systems because of the anatomy and physiology of the vascular system; it supplies blood and nutrients to all organs of the body. Drugs can be introduced into the vascular system for systemic effects or targeted to an organ via the regional blood supply. In addition to the usual formulations of drugs such as controlled release, devices are used as well. A considerable amount of cardiovascular therapeutics, particularly for major and serious disorders, involves the use of devices. Some of these may be implanted by surgery whereas others are inserted via minimally invasive procedures involving catheterization. Use of sophisticated cardiovascular imaging systems is important for the placement of devices. This chapter will start with a brief introduction to cardiovascular diseases, historical evolution, and trends in future therapeutics.

## History of Cardiovascular Therapy

Ancient Chinese and Egyptian physicians knew about the heart and the pulse but there was no concept of circulation or treatment of heart disease. The sixteenth century father of anatomy, Andreas Vesalius, first described proper anatomy of the human heart, and the seventeenth century English physician, William Harvey, described the circulation of the blood. A Cambridge-trained physician, William Heberden, provided the classic description of pain due to coronary heart disease and named it angina pectoris. Landmarks in the historical evolution of cardiovascular therapy are shown in Table 1.1.

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**Table 1.1** Landmarks in the historical evolution of cardiovascular therapy

| Year | Landmark |
|---|---|
| 1785 | William Withering discovered foxglove; its extract digitalis became a mainstay for treatment of CHF |
| 1847 | Invention of glyceryl trinitrate by Ascanio Sobrero in Italy and demonstration of headache and other systemic effects after its administration by sublingual route (Marsh and Marsh 2000) |
| 1876 | Introduction of glyceryl trinitrate for the treatment of angina pectoris (Murrell 1879) |
| 1881 | Invention of a device to measure blood pressure by Samuel Siegfried Karl Ritter von Basch of Austria |
| 1929 | Start of the modern era of cardiac catheterization: Werner Forssmann's dramatic right-heart self-catheterization in an effort to find ways to inject drugs into the heart for cardiac resuscitation |
| 1960 | Coronary artery bypass graft procedure introduced |
| 1960 | First description of myocardial ischemia-reperfusion injury (Jennings et al. 1960) |
| 1970 | Percutaneous transluminal coronary angioplasty procedure introduced |
| 1974 | Concept of image-guided catheter-directed thrombolysis as an alternative method to systemic intravenous infusion (Dotter et al. 1974) |
| 1980s | Invention of the first stent by Julio Palmaz |
| 1985 | First proposal for cell therapy of cardiovascular disorders: myocyte transplantation for treatment of complete heart block (Sade and Fitzharris 1985) |
| 1989 | Pulsed-spray pharmacomechanical thrombolysis (Bookstein et al. 1989) |
| 1990 | Introduction of the drug-eluting stents |
| 1995 | Gene therapy for cardiovascular disease: first clinical trial of familial hypercholesterolemia using ex vivo hepatic low-density lipoprotein receptor gene transfer via a retroviral vector |
| 2000 | Local drug delivery by regional myocardial infiltration by the percutaneous coronary venous route demonstrated in experimental animals (Herity et al. 2000) |
| 2000 | Introduction of induced angiogenesis therapy for myocardial ischemic disease |
| 2002 | Transplantation of hematopoietic stem cells into patients for treatment of ischemic heart disease |

## Overview of Cardiovascular Disease

### *Epidemiology of Cardiovascular Disease*

Cardiovascular disease (CVD) has remained the leading cause of death worldwide despite the tremendous progress made in medical and surgical treatment for this disease. In the USA, approximately 70 million persons have symptoms or findings (without symptoms) pertaining to coronary artery disease (CAD); of these patients, 10% have clinically confirmed disease. Not all of these patients have coronary artery disease, as some may have only angina pectoris but no demonstrable pathology in the coronary arteries. On the other hand, there are patients with coronary artery disease who may not have any investigations or require hospitalization. Sometimes it is postmortem finding in patients who die of other causes. The incidence is 1 million myocardial infarctions per year and 700,000 coronary-related deaths per year in the USA. Nearly 8 million Americans alive today have suffered

at least one heart attack and so are at greater risk for congestive heart failure (CHF) or another, potentially fatal, heart attack. Each year, of more than 1.2 million Americans who suffer heart attacks, about 400,000 develop CHF due to damage to the heart, and half of these die within 5 years. People who have had a heart attack have a sudden death rate that is 5–6 times greater than in the general population. Hypertension affects about 70 million persons in the USA with an overlap with those suffering from CAD.

The prevalence of peripheral arterial disease (PAD) in the general population has been estimated to approach 12% in the USA. About one third of patients with CAD also suffer from PAD. The most common cause of PAD is arteriosclerosis obliterans – segmental arteriosclerotic narrowing or obstruction of the lumen of the arteries supplying the limbs. The lower limbs are involved more frequently than the upper limbs. Although not life threatening, this condition causes considerable pain and disability.

There is overlap in the patient populations with various cardiovascular disorders. For example, hypertension is a risk factor for heart disease and can be found in patients with CAD. Most of the myocardial ischemia is due to angina pectoris but some of these patients have myocardial infarction. CHF can occur in patients with myocardial infarct.

## *Management of Acute Coronary Occlusive Disease*

Acute coronary occlusion is usually accompanied by a myocardial infarct (MI) with symptoms such as chest pain radiating to the arm and shortness of breath. Silent MI may be asymptomatic. The aim of management is to salvage as much myocardium as possible and to prevent further complications. Nitroglycerine can be given to relieve chest pain, and antiplatelet agents such as aspirin are used.

Platelets play a central role in the pathophysiology of acute coronary syndromes and activation of platelet glycoprotein (GP) IIb/IIIa receptor is critical to platelet aggregation. Abciximab, a human murine chimeric antibody to the GPIIb/IIIa receptor, is an important biological therapy in the management of patients presenting with acute coronary syndromes. An adverse effect of abciximab is thrombocytopenia. Abciximab was a breakthrough drug in the management of high-risk patients undergoing percutaneous coronary intervention (PCI). However, with newer available therapies and improvement in PCI technology, dose and delivery of this drug have evolved as we try to extract maximum benefit while minimizing the adverse effects (Parikh and Juergens 2011).

## *Limitations of Current Therapies for Myocardial Ischemic Disease*

Since the purpose of DDS is to improve cardiovascular therapeutics, some examples of challenges and limitations of currently available cardiovascular therapeutics should be noted. The most common procedure for coronary artery occlusive disease, stenting, is associated with restenosis, which will be discussed later in this chapter.

Despite major advances in diagnosis and prevention of heart disease, heart attacks continue to be the major cause of morbidity and mortality in the industrialized world. A heart attack, due to blocking of coronary artery, acute myocardial infarction, has serious consequences. Within minutes, lack of oxygen in the tissue irrigated by the coronary artery, causes cell death in the heart muscle. If thrombolytic treatment is not administered immediately, the damage is irreversible. The adult heart lacks reserve cells and cannot regenerate. Reperfusion injury including no-reflow phenomenon may increase the infarct size and require cardioprotective strategies that are mentioned later in this chapter. In the postinfarction phase, the remodeling process is characterized by hypertrophy of myocardium and dilation of the left ventricle with cardiomyocyte replacement by fibrous tissue. There is progressive loss of viable tissue, and infarct extension eventually results in heart failure.

Current therapeutic options for myocardial infarction include medical therapy of proven but limited benefit, and various surgical options, which have either restricted applicability or unproven benefit. Various methods of treating heart failure include long-term medication, temporary devices such as artificial pumps, and heart transplants. Less than one in ten persons who need heart transplants will find suitable donors. Although the current treatments for cardiovascular disease prevent heart attack from occurring and/or alleviate its aftereffects, they do not repair the damaged muscle that results, leaving sizably dead portions of heart tissue that lead to dangerous scars in the heart. Damage done by a heart attack to heart muscle is really the cause of all the serious complications of the disease: disturbances of heart rhythm can lead to sudden cardiac death and decreased muscle pumping function can lead to congestive heart failure. The ideal treatment is to repair the damage done to the heart muscle and prevent these complications. This is the rationale for cell/gene therapies to be described in later chapters of this report.

## *Angina Pectoris*

Angina pectoris is a serious and debilitating heart condition marked by repeated and sometimes unpredictable attacks of cardiac pain or discomfort. Angina attacks are typically triggered by physical exertion or emotional stress and occur when the heart is not receiving all the oxygen that it needs to function effectively. Usually angina is associated with coronary artery disease, which is characterized by a buildup of fatty plaques in coronary arteries that reduce the flow of oxygen-rich blood through the heart. When the blood supply to the heart is inadequate and cannot provide enough oxygen to meet the heart muscle's demand (a condition called myocardial ischemia), an angina attack may occur.

## *Cardiomyopathies*

Cardiomyopathies are disorders affecting the heart muscle that frequently result in congestive heart failure. Five major forms are recognized: dilated, hypertrophic,

restrictive, right ventricular, and nonclassifiable cardiomyopathies with distinct hemodynamic properties. Furthermore, the new WHO/WHF definition also comprises inflammatory cardiomyopathy, defined as myocarditis in association with cardiac dysfunction. Idiopathic, autoimmune, and infectious forms of inflammatory cardiomyopathy were recognized. Viral cardiomyopathy is defined as viral persistence in a dilated heart. In recent years, there have been breakthroughs in understanding the molecular and genetic mechanisms involved in this group of conditions, enabling improvement of diagnostic strategies and introduction of new therapies. Ongoing evaluation of antiviral, immunoglobulin, removal of antibodies by immunoadsorption, anticytokine and gene therapy, as well as the mechanical support devices may provide new treatment options.

## *Cardiac Arrhythmias*

The normal cardiac rhythm originates in the sinoatrial (SA) node, a patch of cells called the pacemaker, which generates cardiac rhythms for coordinated contractions and blood pumping. Accelerated transmission of electrical impulses through the atrioventricular (AV) node, a critical regulator of heart rate, is largely responsible for the rapid heart rate during many atrial arrhythmias, such as atrial fibrillation, atrial flutter, and paroxysmal atrial tachycardias. Prompt slowing of AV nodal conduction is often the immediate goal of treatment to slow the abnormally rapid heart rate. Atrial arrhythmias are potentially life-threatening situations with such consequences as stroke, heart attack, and low blood pressure, and require immediate treatment. Caffeine, tobacco and stress may trigger an increase in the speed of conduction through the AV node resulting in atrial arrhythmias.

Cardiac arrhythmias are a leading cause of morbidity in the Western hemisphere. The risk of developing malignant ventricular tachyarrhythmias is associated with the extent of myocardial injury and is believed to be the primary cause of approximately 50% of all cardiovascular deaths. Bradycardia and heart block, which can result from the normal aging process, further add to the morbidity associated with cardiac arrhythmias and results in the permanent implantation of over 160,000 pacemakers annually in the USA.

Conventional medical therapy is predominantly palliative treatment and commonly fails to impede and prevent the morbidity and mortality associated with cardiac arrhythmias. Radiofrequency catheter ablation of ischemic ventricular tachycardia is considered adjuvant therapy rather than curative. The implantation of defibrillators and pacemakers, while generally effective, do have problems which include: (1) implantation of a mechanical device and its need for replacement every 4–7 years, (2) surgical and mechanical complications resulting from the implantation of the device, (3) negative physical and psychological effects of an implanted mechanical device, (4) a prevalent need to use concurrent antiarrhythmic therapy and/or radiofrequency modulation/ablation, and (5) a relatively high cost. Therefore, there is a need to develop alternative therapies for treatment of conduction abnormalities that overcomes the negative aspects of current treatment methods.

## *Congestive Heart Failure*

Congestive heart failure (CHF) occurs when the heart muscle is weakened by disease and cannot adequately pump blood throughout the body. The most common symptoms of heart failure are due to fluid overload: shortness of breath; feeling tired; and swelling in the ankles, feet, legs, and sometimes the abdomen. Among other problems, reduced blood flow can impair the ability of the kidneys to clear fluid waste from the body. There is no cure for CHF, but furosemide is still the mainstay of treatment of congestion in patients with CHF. Shortcomings of furosemide treatment include the development of resistance and side effects such as electrolyte abnormalities, neurohormonal activation, and worsening renal function. Alternative treatments include loop diuretics, combined diuretic therapy, dopamine, inotropic agents, ultrafiltration, natriuretic peptides, vasopressin, and adenosine antagonists. There are few controlled studies to assess treatments for overcoming resistance to furosemide and to protect the kidney from its untoward effects, and the results have been mostly inconclusive, indicating a need for finding better treatments of congestion in heart failure (Metra et al. 2011).

The ability of the heart to pump blood is determined by the movement of cytosolic $Ca^{2+}$ in and out of an organelle in the heart cells called the sarcoplasmic reticulum. Malfunction in sarcoplasmic reticulum ATPase2a (SERCA2a) pathway, an important regulator of myocardial contractility, is associated with progressive heart failure. Therapeutic agents which modulate the activity of SERCA2a have beneficial effects on cardiac contractility and prevent the progression of heart failure.

## *Peripheral Arterial Disease*

The commonest cause of peripheral arterial disease (PAD) is arteriosclerosis obliterans, which denotes segmental arteriosclerotic narrowing or obstruction of the lumen of the arteries supplying the limbs. It becomes manifest between the ages of 50 and 70 years. The lower limbs are involved more frequently than the upper limbs. Thromboangiitis obliterans is an obstructive arterial disease caused by segmental inflammatory and proliferative lesions of the medium and small vessels of the limbs. The etiology is unknown, but there is a strong association with cigarette smoking and autoimmune factors. There may be a genetic disposition to this disease and it is most prevalent between the ages of 20 and 40 years. Systemic diseases such as diabetes mellitus may be associated with PAD. Sudden occlusion of an artery to a limb may result from an embolus or thrombosis in situ. It occurs in 10% of cases of arteriosclerosis obliterans, but it is rare in thromboangiitis obliterans. The heart is the most frequent source of emboli in this syndrome; they may arise from a thrombus in the left ventricle.

Limb pain is the most frequent symptom of PAD. In the case of the legs, calf pain appears on walking a certain distance and disappears on rest. This is referred to as intermittent claudication. Pain at rest is a sign of severe PAD and occurs when there is a profound reduction in the resting blood flow to the limb. In sudden arterial

occlusion, there may be numbness and weakness of the affected limb as well. The arterial pulses distal to the site of obstruction are lost or reduced. The skin temperature is low and there may be pallor or reddish blue discoloration. There may be ulceration or gangrene of the affected extremity.

Reduction of blood flow to a limb is due to stenosis of the arteries. Stenosis that decreases the cross-sectional area of an artery by less than 75% usually does not affect the resting blood flow to the limb; lesser degrees of stenosis may induce muscle ischemia during exercise. The presence or absence of ischemia in the presence of obstruction is also determined by the degree of collateral circulation. Some collateral vessels that are normally present do not open up until the obstruction occurs and may take several weeks or months to be fully developed.

Treatment of PAD is not satisfactory. Endarterectomy, where the thrombus is removed, is usually done in acute occlusion. This procedure is less successful in small vessel occlusion. Where the stenotic lesion cannot be corrected, vein bypass or excision and replacement with a synthetic graft may be tried. Percutaneous balloon angioplasty is quite popular although the long-term results are controversial. Laser angioplasty and radiofrequency angioplasty are promising new techniques. Stents are also placed in the arterial lumen and restenosis is a problem with all the procedures.

## *Cholesterol and Atherosclerosis*

Atherosclerosis is a disease of the vessel wall involving lipid accumulation, chronic inflammation, cell death, and thrombosis that causes heart disease and stroke. Although elevated cholesterol levels are a recognized risk factor for atherosclerosis, a growing number of studies now suggest that oxidized phospholipids may also play an important role in this condition. Phospholipids, essential components of lipoproteins and cell membranes, are susceptible to free radical or enzymatic oxidation by myeloperoxidase, lipoxygenase, and other enzymes that are present in the vessel wall. Statins are the main drugs used currently to lower cholesterol but it is likely that other therapies such as those directed against oxidized phospholipids will be developed in the next decade.

High-density lipoprotein (HDL) may provide cardiovascular protection by promoting reverse cholesterol transport from macrophages, but accounted for less than 40% of the observed variation. Cholesterol efflux capacity from macrophages, a measure of HDL function, has a strong inverse association with both carotid intima-media thickness and the likelihood of angiographic coronary artery disease, independently of the HDL cholesterol level (Khera et al. 2011).

## *Familial Hypercholesterolemia*

Familial hypercholesterolemia (FH) is an autosomal dominant disorder resulting from a defect in the gene for low-density lipoprotein (LDL) receptors with resulting impairment of metabolism of cholesterol. The defect is common: approximately

1 in 500 persons is a heterozygote. Homozygotes are extremely rare with an incidence of 1 in one million. Patients with hypercholesterolemia manifest skin deposits of cholesterol called xanthomas and atherosclerosis. Most of the untreated patients die of coronary artery disease at a young age. Current therapy is a combination of diet to decrease cholesterol intake and statins. However, a number of patients are still failing to reach treatment guidelines even with the most effective of the currently available statins.

### *The Endothelium as a Target for Cardiovascular Therapeutics*

The endothelium is a complex organ system that controls the homeostasis of the vasculature by integrating signals between the vascular wall and the vessel lumen. Under physiological conditions, it maintains a normal vascular tone and blood fluidity by elaborating a variety of factors, such as nitric oxide, prostacyclin, and endothelin. Disturbances of the endothelium can produce vasoconstriction, inflammation, and thrombotic events and manifest in various disorders such as hypercholesterolemia and hypertension. There are several causes of altered endothelial functions and the mechanisms underlying them are complex and not yet fully understood. There is substantial evidence that many endothelial functions are sensitive to the presence of reactive oxygen species and subsequent oxidative stress. Exogenous antioxidants can modulate the endothelium-dependent vasodilation responses, the endothelium–leukocyte interactions, the balance between pro- and antithrombotic properties, and the vascular apoptotic responses.

## Molecular Cardiology

Advances in genomics and achievements of the Human Genome Project had an enormous impact on medicine, giving rise to the term genomic medicine. This has also changed the classic practice of clinical cardiology in many ways, increasing our awareness of inheritance of defective genes and their impact on health and disease, and providing new diagnostic and therapeutic tools as discussed under cadiogenomics. Proteomics has a further impact on cardiology and will be discussed under cardioproteomics.

The cellular and molecular mechanisms underlying cardiovascular dysfunctions are mostly unknown. Molecular cardiology, as a part of molecular medicine, involves the use of new biotechnology tools to gain an understanding of the cardiovascular function and the pathways involved in disease. This provides the foundation for rational and innovative approaches to cardiovascular therapeutics.

## *Cardiogenomics*

The term cardiogenomics refers to the role of genes in cardiovascular system. Microarrays are being applied to study gene expression. The patterns in the variation of expression of many genes correlate well with the models currently used to explain the pathogenesis of cardiovascular diseases.

More complete genomic maps allow easier identification of genes that cause monogenic inherited diseases. In addition, analyses of variations in gene expression in cardiovascular diseases are revealing new potential candidate genes as well as novel biomarkers for many common, multifactorial diseases. While experiments are revealing new pathophysiologic pathways, these genomic studies are also generating enormous amounts of data which is being analyzed with bioinformatics techniques. Genomics is influencing the approaches of treatment and prevention of cardiovascular diseases, and this will be discussed further under pharmacogenomics of cardiovascular disorders.

### Genomics of Dilated Cardiomyopathy

Dilated cardiomyopathy (DCM) is characterized by impairment of cardiac contractile function leading to cardiac failure. The molecular basis of idiopathic dilated cardiomyopathy is largely unknown but multiple etiological factors are involved. SeqWright Inc, in collaboration with Roche and the University of Miami's Miller School of Medicine, is conducting research to identify possible genetic variants associated with DCM by using NimbleGen Sequence Capture Human Exome Arrays to enrich over 180,000 exons from DNA samples from individuals affected with this disease. SeqWright is sequencing the enriched exons to detect genetic variants within these samples, including single nucleotide polymorphisms (SNPs) and insertions and deletions.

## *Cardioproteomics*

Proteomics is the systematic analysis of protein profiles of tissues and the term "proteome" refers to all proteins produced by a species, much as the genome is the entire set of genes. Unlike the genome, the proteome varies with time and is defined as "the proteins present in one sample (tissue, organism, cell culture) at a certain point in time." Proteomics parallels the related field of genomics. Proteomic technologies are described in a special report on this topic (Jain 2011). Proteomics provides a set of tools for the large-scale study of gene expression at the protein level, thereby allowing for the identification of protein alterations responsible for the development and the pathological outcome of diseases, including those of the cardiovascular system. The term cardioproteomics refers to the use of proteomic technologies for the study of cardiovascular system.

Evolution of proteomic techniques has permitted more thorough investigation into molecular mechanisms underlying cardiovascular disease, facilitating identification not only of modified proteins but also of the nature of their modification. An example is heart disease resulting in heart failure, which is among the leading causes of morbidity and mortality in the developed world. The current treatments provide only symptomatic relief and there is need for rational cures. The pathomechanism of underlying cardiac dysfunctions in heart failure are largely unknown but is likely associated with significant myocardial gene and protein expression abnormalities. A characterization of these changes would help in understanding the pathomechanism of cardiac dysfunction and provide new diagnostic markers and therapeutic opportunities. Proteomics has the potential to characterize alterations in protein expression in cardiac failure. Proteomics supplements genomics-based and other traditional approaches to the investigation of cardiac disorders.

### Pathomechanism of Cardiovascular Diseases

The pathomechanism of underlying cardiac dysfunctions in heart failure are largely unknown but is likely associated with significant myocardial gene and protein expression abnormalities. A characterization of these changes would help in understanding the pathomechanism of cardiac dysfunction and provide new diagnostic biomarkers and therapeutic opportunities. Proteins of particular interest are those involved in the induction of cardiac (mal) adaptive hypertrophic growth, interstitial fibrosis, and contractile dysfunction. Proteomics enables pathophysiological questions to be approached exclusively from the protein perspective, and may enable us to map the entire complement of proteins expressed by the heart at any time and condition (Faber et al. 2006). Proteomics supplements genomics-based and other traditional approaches to the investigation of cardiac disorders. This approach creates the unique possibility to identify, by differential analysis, protein alterations associated with the etiology of heart disease and its progression, outcome, and response to therapy.

Protein alterations in cardiovascular disease include those that are suitable candidates for drug targets and diagnostic disease biomarkers as well as therapeutic proteins/peptides. Since gene therapy depends on the function of a therapeutic protein encoded by a “therapeutic” gene, proteomic analyses also provide the basis for the design and application of gene therapies. Proteomic technologies enable not only identification of proteins but also the nature of their posttranslational modifications thus enabling the elucidation of signal transduction pathways and their deregulation under pathological conditions. The linkage of information about proteome changes with functional consequences lead to the development of functional proteomic studies. Functional proteomic analyses will improve our understanding of the relations between proteome changes and cardiovascular dysfunctions.

### Study of Cardiac Mitochondrial Proteome in Myocardial Ischemia

Myocardial ischemia-reperfusion induces mitochondrial dysfunction and, depending upon the degree of injury, may lead to cardiac cell death. However,

ability to understand mitochondrial dysfunction has been hindered by an absence of molecular biomarkers defining the various degrees of injury. As an attempt to characterize the impact of ischemic damage on mitochondrial proteome biology, an in vitro model of cardiac mitochondria injury in mice was established to examine two stress conditions: reversible injury induced by mild calcium overload and irreversible injury induced by hypotonic stimuli (Zhang et al. 2008). Both forms of injury had a drastic impact on the proteome biology of cardiac mitochondria. Altered mitochondrial function was concomitant with significant protein loss/shedding from the injured organelles. In the setting of mild calcium overload, mitochondria retained functionality despite the release of numerous proteins, and the majority of mitochondria remained intact. In contrast, hypotonic stimuli caused severe damage to mitochondrial structure and function, induced increased oxidative modification of mitochondrial proteins, and brought about detrimental changes to the subproteomes of the inner mitochondrial membrane and matrix. Key observations made by the in vitro model were validated by using an established in vivo murine model of regional myocardial ischemic injury. This preclinical investigation provides function and suborganelle location information on a repertoire of cardiac mitochondrial proteins sensitive to ischemia reperfusion stress, and highlights protein clusters potentially involved in mitochondrial dysfunction in the setting of ischemic injury.

### Cardiac Protein Databases

HEART 2D PAGE database of human cardiac proteins have been established at the German Heart Institute, Berlin: http://userpage.chemie.fu-berlin.de/~pleiss/dhzb.html (accessed 3 Jan 2011). It contains data on proteins identified on the 2D PAGE maps of ventricle and atrium of human heart. It contains protein expression characteristics for patients suffering from dilated cardiomyopathy (DCM).

### Proteomics of Dilated Cardiomyopathy and Heart Failure

Mutations in sarcomere protein genes account for approximately 10% of cases of familial DCM. Several studies have shown that expression of about 100 cardiac proteins is significantly different from normal in DCM and most of these proteins are less abundant in the diseased than in the normal heart. Impairment of expression of several cardiac proteins in DCM can be demonstrated by 2DGE. Many of these proteins have been identified by chemical methods and are classified into three functional classes:

- Cytoskeletal and myofibrillar proteins
- Proteins associated with mitochondria and energy production
- Proteins associated with stress response such as heat shock protein 27

Correlation of the changes in proteins to altered cellular function is a challenge. Studies on samples from human diseased tissues are difficult to interpret because of large number of variables. An alternative approach is animal models of human

disease. Several models of cardiac hypertrophy are available in small laboratory animals such as the rat, and proteomic studies have been done in large animal models such as bovine DCM.

The comparison of 2DGE patterns from DCM patients with those of controls has revealed statistically significant intensity differences which forms the basis of a human myocardial 2DE database. The number of proteins that can be identified is limited only by the capacity of a laboratory and the personnel rather than by the principle of feasibility. Specific proteins are hyperubiquitinated in diseased hearts. These proteins can be purified by affinity chromatography and identified using 2D PAGE/MALDI-TOF. The ubiquitin-proteosome pathway is a possible target for therapeutic intervention in heart disease.

### Role of Proteomics in Heart Transplantation

Heart transplantation is now an established procedure for treating patients with end-stage heart failure. During the past 20 years, the number of heart transplants performed worldwide has increased from a few to approximately 4,000 per year. Major problems following heart transplantation include infection with cytomegalovirus (CMV) and rejection – hyperacute, acute, or chronic. Hyperacute rejection is the failure or rejection of graft with the first 24 h. Acute rejection is the major risk for survival in the first posttransplant year. It is usually monitored by regular endomyocardial biopsy and histological examination. One-year survival in heart transplantation is 85%, and 50% of the grafts are lost after 5 years.

Proteomics is being used to develop noninvasive methods for detecting acute rejection after cardiac transplantation. Mass spectrometry analysis identifies a set of proteins in the heart altered with acute or chronic rejection. ELISA (enzyme-linked immunosorbent assays) have identified proteins in serum samples of patients that are potential noninvasive biomarkers of acute and/or chronic rejection. Validation of these diagnostic markers requires analysis of larger sets of patient and serum samples. Early detection of rejection is important as it provides an opportunity for treatment with immunosuppressant therapy.

### Future of Application of Proteomics in Cardiology

Proteomic analysis has provided important insights into ischemic heart disease, heart failure, and cardiovascular pathophysiology. The future holds great promise for the availability of a panel of cardiac serum biomarkers able to delineate different stages of each heart disease, thus allowing the design of clinical interventions potentially using stage-specific therapeutics. All of this is feasible only with detailed information about the unique and selective protein modifications that occur during the development of heart disease. The combination of proteomic biomarkers with clinical phenotypes and genetic haplotype information can lead to a more precise diagnosis and therapy of heart disease on an individual basis – personalized cardiology (Arab et al. 2006).

## *Ion Channels and the Cardiovascular System*

Ion channels of major interest in the cardiovascular system are those that mediate $Na^+$, $K^+$, and $Cl^-$ transport. They are of critical importance in generating conducting signals and action potentials which elicit the physiologically essential function of contractile cardiac and vascular smooth muscle. Paradigms of cardiovascular physiology and pathophysiology can be summarized as follows:

1. From a cellular biochemical point of view, regulation of cardiac work is done by changing myocardial contractility and calcium fluxes. During heart failure, there is impaired myocardial contractility and relaxation. Electrophysiological basis of this is abnormal ionic currents and altered channel function.
2. From a gene expression point of view, regulation of cardiac work is based on altered synthesis of myocardial proteins. During cardiac failure, there are myocardial alterations and cardiomyopathy of overload. The electrophysiological basis of this is altered ion channel synthesis and assembly.

### Cardiac Rhythm Disturbances due to Ion Channels

Sudden cardiac death is a major cause of mortality in the industrialized world. These deaths are usually due to cardiac rhythm disturbances as a result of altered electrophysiology. Persons with diseased hearts are more prone to cardiac rhythm disturbances than those with healthy hearts. Sudden cardiac death involves an interaction between structural derangement of the heart, transient functional disturbances, and specific electrophysiological events responsible for fatal arrhythmia. Various arrhythmias following myocardial infarction involve some form of reentry mechanism. Atrial and ventricular fibrillation are based on multiple interlacing wavelets of reentry. Arrhythmias such as torsade de pointes and brief polymorphic form of ventricular tachycardia seen during acute-phase infarction and in patients with heart failure appear not to be due to reentry but perhaps are caused by triggered automaticity. Similar electrophysiological disturbances are likely to occur in global dilated cardiomyopathy and these are more widespread than in regional damage due to myocardial infarction. In such patients, measures should be directed at the basic disease. There is no evidence that prophylactic antiarrhythmic therapy is of benefit in these patients.

### Gap Junction and Cardiovascular Disease

Electrical coupling between cardiac muscle cells is mediated by specialized sites of plasma membrane interaction termed gap junctions. In ischemic heart disease, the orderly distribution of the gap junctions is lost in the zone border of the myocardial infarct. There is reduction in the number of connexin 43 gap junctions. These and other changes in the gap junction reduce conduction velocity and predispose to arrhythmias.

Gap junctions could play a role in a diverse number of vascular diseases in which primary alterations in the smooth muscle or endothelial cells are considered to be significant factors. A partial list of vasculopathies that might be associated with altered intercellular communication includes the following:

- Coronary vasospasm
- Raynaud's syndrome
- Essential hypertension
- Cardiovascular complications of diabetes mellitus

### Ion Channels and Hypertension

The regulation of vascular tone, and hence blood pressure, is under the control of a variety of ion channels in vascular smooth muscle cells (VSMCs). Two types of ion channels are perhaps most important in determining the contractile state of VSMCs: $K^+$ channels, which are primary determinants of the resting membrane voltage, and voltage-gated L-type $Ca^{2+}$ channels, activation of which allows $Ca^{2+}$ influx and vasoconstriction.

A number of different K+channel subtypes are expressed in VSMCs, including several members of the KCNQ (Kv7) family of voltage-gated K+channels. Kv7.1–Kv7.5 channels are encoded by five genes (KCNQ1–5). Pharmacological alteration of the activity of these channels has dramatic effects on VSMC excitability, contraction and vascular tone. The activity of vascular Kv7.5 channels is suppressed by the pituitary hormone arginine vasopressin (AVP) to mediate its physiological vasoconstrictor effects.

Calcium-dependent $K^+$ efflux is increased in arteries from genetic and renal rat models of established hypertension. It has been suggested that this enhanced $K^+$ current is a consequence of high blood pressure and is a compensatory mechanism activated during the development of hypertension to counteract arterial excitability and prevent further vasoconstriction. This hypothesis is supported by experimental findings that $Ca^{2+}$-dependent $K^+$ current density in arterial muscle membrane has a positive correlation with chronic arterial blood pressure levels.

$Na^+/K^+$ exchange mediates growth factor-induced smooth muscle cell proliferation. Defect in this exchanger activity may be linked to hypertension, vascular hyperplasia, and atherosclerosis. Cultured smooth muscle cells of spontaneously hypertensive rats exhibit an increase in proliferation and also an elevated $Na^+/K^+$ exchange activity compared with vascular smooth muscle cells of normotensive Wistar rats. Similar activity is seen in lymphocytes and other blood cells from patients with essential hypertension and this may be related to de novo synthesis of exchanger proteins.

Many reports support the idea that factors that inhibit the plasma membrane Na+, K+−ATPase are involved in certain forms of hypertension. One of the factors, the hypothalamo-pituitary inhibitory factor, is an endogenous compound which inhibits

the calcium pump of the synaptosomal plasma membrane by disruption of the lipid annulus. This inhibition could play a role in the control of calcium homeostasis by increasing the cytosolic free calcium concentration. The relevance of this effect on synaptic hyperactivity and increased tone of the vascular smooth muscle is under investigation.

Among the multiple factors hypothesized to play a role in the pathogenesis of hypertension, the following are relevant to ion channels and transporters:

- A defect in the L-type $Ca^{2+}$ channels lead to rise of intracellular calcium.
- An increase in membrane permeability leading to rise of intracellular sodium.
- Inhibition of sodium transport by an increase in natriuretic hormone induced by high sodium intake and stress.

Drugs that target $Ca^{2+}$ and $K^{+}$ channels are used clinically as antihypertensive medications.

## Ion Channels and Congestive Heart Failure

There is sufficient evidence that $Ca^{2+}$ handling is defective in the failing heart. In dilated and hypertrophic cardiomyopathy, there are both fast and slow $^{Ca2+}$ transients compared to the single fast transient observed in the control tissue. The plateau phase of the action potential is prolonged and tension relaxation is slowed. These observations suggest a defect at the level of $Ca^{2+}$ entry or storage. There is evidence for the downregulation of L-type $Ca^{2+}$ channels in the canine experimental model of congestive heart failure. The downregulation of β-adrenoreceptors observed in both the canine and rodent systems parallels those found in patients suffering from end-stage heart failure.

## $K_{ATP}$ Channels and Myocardial Disease

Myocardial disease and ischemia can alter the electrophysiological function of ion channels responsible for the cellular electrical activity of the heart, particularly that of the inward- and outward-rectifier potassium channels. Abnormalities of the membrane function favor the development of slow conduction and unidirectional block, which are essential for establishing a reentry pathway capable of supporting ventricular tachyarrhythmias. The accompanying decrease in tissue ATP content, increase in tissue free fatty acids, and gain of intracellular sodium and calcium ions each activate separate potassium channels. $K_{ATP}$ channel in the heart is active only in pathological circumstances where it can play a potentially crucial role in the genesis of fatal reentrant arrhythmias. Functionally, active $K_{ATP}$ channels become manifest only in myocardial cells in which ATP is decreased. This concept is supported by the finding that glibenclamide, a KATP blocker reverses

the electrophysiological consequences of ischemia in isolated myocardial cells. Glibenclamide is also effective in preventing the development of ventricular fibrillation in isolated heart preparations under conditions of low intracellular ATP. Potassium loss during ischemia/hypoxia is attributed to activation of $K_{ATP}$ channel and this is partially blocked by the action of glibenclamide. Many of the sequelae of metabolic stress in the cardiac muscle are related to loss of sensitivity of these channels to glibenclamide as observed in the canine model of congestive heart failure. The underlying mechanism of this is not known but a reduction in sensitivity of these channels contributes to postinfarct electrophysiologic disturbances of the heart.

#### Long Q-T Interval Syndrome as an Ion Channel Disorder

Long Q-T interval syndrome (LQTS) is an inherited cardiac disorder characterized by a prolonged Q-T interval on ECG due to slow repolarization of the action potential. Individuals with this syndrome are at risk of sudden death due to ventricular arrhythmias. Mutations of the human HERG (ether-a-go-go related gene), an inward rectifier potassium channel have been shown to cause a form of LQTS.

### *Role of Plasminogen Activator Inhibitor-1 in the Cardiovascular System*

Plasminogen activator inhibitor-1 (PAI-1) is critical in thrombus formation and inflammation and plays a role in the progression of various cardiovascular diseases. To date, a novel PAI-1 inhibitor have been reported to suppress the development of experimental autoimmune myocarditis, vascular remodeling after arterial injury, and heart transplant rejection using rodent models (Suzuki et al. 2011). Pathologically, the PAI-1 inhibitor improved histological remodeling of myocardium and arteries with suppression of inflammation and thrombus formation. Thus PAI-1 inhibitors appear to exhibit potent effects on the prevention of adverse tissue remodeling. However, PAI-1 is a multifunctional protein and more research is needed to further elucidate the association between PAI-1 expression and cardiovascular disease.

## Biotechnology and Therapy of Cardiovascular Diseases

Optimal use of existing therapeutics and discovery of new treatments for cardiovascular disorders would involve improved methods of biotechnology. Interrelationships of drug biotechnology and therapy of cardiovascular diseases are shown in Fig. 1.1.

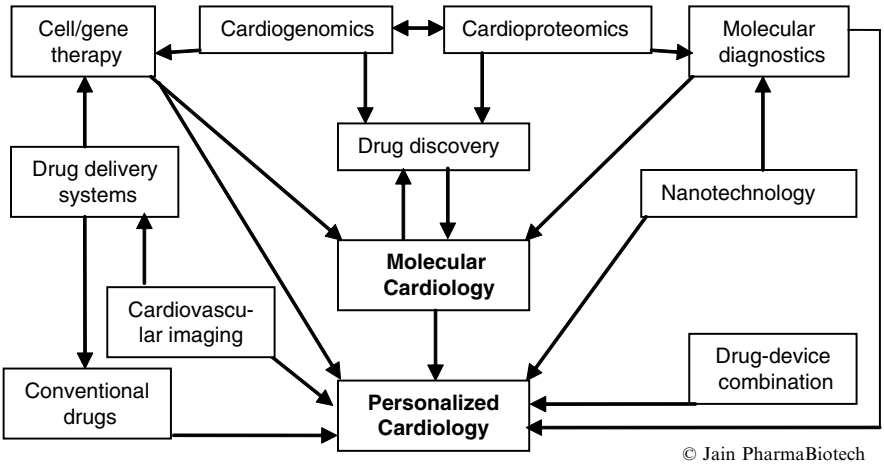


**Fig. 1.1** Biotechnology and therapy of cardiovascular diseases

## Chronopharmacotherapy of Cardiovascular Diseases

The clinical importance of circadian biological rhythms has been strengthened by a number of studies showing a circadian distribution of cardiovascular events like myocardial infarction, stroke, complex arrhythmia, or sudden cardiac death. Incidence of diseases showed a maximum during the early morning hours after awakening from sleep. In addition, a number of pathophysiological mechanisms has been identified to coincide with this peak including blood pressure and heart rate surges, decreased endothelial dilatory capacity of peripheral and coronary arteries, enhanced sympathetic activity, decreased cardiac electrical stability, and increased platelet aggregation. This time window of high risk for the incidence of cardiovascular events has been identified as a target for new treatment and prevention strategies including new release forms of antihypertensive and coronary-dilatory drugs.

Chronotherapeutics is the delivery of a medication in a dosage form to provide optimal levels at the time of maximal need, which vary according to daily rhythms. Circadian variations have been established in the following cardiovascular diseases:

*Hypertension*. In most hypertensive patients, there is a fairly marked rise in blood pressure (BP) upon awakening that is called the morning or "AM" surge. BP usually declines in the afternoon and reaches its lowest point between 12 midnight and 3 a.m.

*Myocardial infarction*. Epidemiological studies have shown that myocardial infarction occurs at least three times more frequently in early morning than in late evening.

*Cardiac arrhythmias*. There is a peak frequency of ventricular tachycardia or fibrillation between 6 a.m. and noon. Arrhythmias are usually suppressed during sleep.

A major objective of chronotherapy is to deliver the drug in the highest concentration during the time of greatest need, for example, early morning hours. Chronotherapeutic agents for hypertension and angina pectoris have been marketed to match drug delivery to the circadian BP and myocardial ischemic rhythms in a novel delivery form. COER-24 contains verapamil as the active drug in a controlled onset, extended release formulation which has a delay in release followed by extended release for approximately 18 h. When taken at bed time, the delivery system provides optimal drug concentration between 4–5 a.m. and noon.

An international study, Controlled ONset Verapamil INvestigation of Cardiovascular End-point (CONVINCE) trial showed that chronopharmacotherapy can reduce the incidence of myocardial infarction and stroke that closely follow fluctuations in BP. Chronotherapy has not yet been recommended as a therapy of choice by various health organizations such as the WHO or the US Joint National Committee V or VI. Comparative trials are still needed to evaluate the effects of chronotherapeutic versus conventional antihypertensive or anti-ischemic therapies.

## Cardioprotection

Apart from the common and the best known cause of myocardial damage due to ischemic heart disease and ischemic/reperfusion injury, several other factors can cause damage to the heart, indicating the need of strategies for cardioprotection. These include cardiac surgery, viral diseases, cardiotoxic chemotherapeutics, atherosclerosis of smaller coronary artery branches, environmental toxins such as carbon monoxide, hypoxia, and aging.

Strategies for cardioprotection are an important part of preventive cardiology and include pharmaceutical as well as non-pharmaceutical measures as shown in Table 1.2. Those relevant to biotechnology are described in this book. Details of other measures for cardioprotection are beyond the scope of this book.

The first strategy in cadioprotection is to remove the causes of ischemic heart disease; the second is to attenuate ongoing ischemic and reperfusion injury; the third is to prevent the progression of cardiac remodeling and chronic heart failure following ischemic injury. For prevention of acute myocardial infarction, it is widely accepted to treat high-risk patients with aspirin and/or statins. On the other hand, several medications such as angiotensin-converting enzyme inhibitors, aldosterone receptor antagonists, and beta blockers have been used for the prevention of postinfarction heart failure in patients who have suffered from an acute myocardial infarction. However, there is adjunctive drug therapy to reduce infarct size in the acute phase in patients with myocardial infarction. The J-WIND trials suggested that an infusion of human atrial natriuretic peptide in the acute phase and oral administration of nicorandil in the chronic phase of infarction result in a better outcome in patients with a myocardial infarction (Asakura and Kitakaze 2010).

**Table 1.2** Strategies for cardioprotection

| |
|---|
| *Control of risk factors for heart disease* |
| Atherosclerosis |
| Hypercholesterolemia |
| Hypertension |
| *Protection against reperfusion injury* |
| Acadesine: increase local adenosine levels |
| Atrial natriuretic peptide (infusion) |
| Calcium channel blockers |
| Cyclosporine: inhibition of mPTP |
| Ischemic preconditioning |
| Nicorandil: $K_{ATP}$ channel opener and NO donor |
| Nitroprusside: nitric oxide-based therapeutics |
| *Other pharmaceuticals approaches* |
| Angiotensin converting enzyme inhibitors |
| Aldosterone receptor antagonists |
| Antioxidants |
| Antiplatelet agents: aspirin |
| Beta blockers: nebivololum, carvedelol |
| dsRNA analog Poly(I:C) targeting Toll-like receptor 3 |
| High-density lipoprotein (HDL) carrier: ETC-216 |
| Hormones: e.g., growth hormone, testosterone |
| Monoclonal antibodies: e.g. pexelizumab |
| Resveratrol |
| Statins |
| *Biological therapies* |
| Cell therapy |
| Gene therapy |
| *Minimally invasive procedures* |
| Coronary angioplasty and stenting |
| Thrombolysis |
| *Surgical procedures* |
| Coronary bypass operation |
| Surgery under cardioprotective inhalation anesthetics |
| *Non-pharmacological approaches* |
| Caloric restriction |
| Exercise |
| Nutritional measures |
| Hyperbaric oxygen |

Pharmaceutical approaches include those for control of risk factors such as hypertension and atherosclerosis as well as antioxidants, hormones (e.g., testosterone), and nitric oxide-based therapeutics. Non-pharmaceutical approaches include exercise, nutritional measures, and hyperbaric oxygen. Biological therapies include stem cell-based therapies and gene therapy. Surgical procedures include those for myocardial revascularization or for temporary support of circulation and oxygenation, and may be combined with cardioprotective inhalation anesthetics.

Following a condition involving ischemia, the heart has an impaired ability to efficiently metabolize various fuels that use oxygen to produce energy. This decreases muscle efficiency, can lead to cell death, and may prolong patient recovery following surgery. Intravenous dichloroacetate has been claimed to protect heart function by temporarily restoring the lost ability to metabolize glucose once blood flow is restarted. It was evaluated in phase II clinical trials on high-risk geriatric patients undergoing open-heart surgery such as coronary artery bypass to improve outcomes and recovery times. Further development was suspended due to the higher incidence of adverse reactions in the treated group compared to the control group. These adverse events were not seen in earlier phase II trials on lower risk non-geriatric patients.

Some of the innovative methods for cardioprotection require suitable and effective methods for delivery. If vascular supply to the myocardium is impaired, systemically delivered therapeutics may not reach the desired site of action in the myocardium. Injections can be made directly into the myocardium but this is not a practical method for cardioprotection.

## *Management of Ischemic/Reperfusion Injury to the Heart*

Ischemic reperfusion injury (IRI) may occur spontaneously following myocardial infarction or may be iatrogenic such as that resulting from revascularization procedures such as coronary artery bypass graft (CABG). Pathogenesis of IRI involves multiple pathways, including ion channels, reactive oxygen species, intracellular calcium overload, activated neutrophils, platelet aggregation, and endothelial dysfunction (Turer and Hill 2010). Management strategies may require an effective control of IRI injury as it is important to prevent increase of myocardial infarct size. Pharmacological strategies include the use of adenosine and carvedilol.

### Adenosine

Adenosine, a purine nucleoside, is a critical component of ATP. Its concentration rises 100-fold during periods of hypoxia and ischemia. Adenosine is released by ischemic tissue and is an important trigger of ischemic preconditioning. $A_1$ and to some extent $A_3$ receptors of adenosine participate in the intracellular signaling that triggers cardioprotection by activating phospholipase C and/or protein kinase C (PKC) directly. Another signaling cascade at reperfusion involves activated PKC, which initiates binding to and activation of an $A_{2b}$ adenosine receptor. $A_{2b}$ agonists, but not adenosine or $A_1$ agonists, infused at reperfusion can initiate this second signaling cascade and mimic preconditioning's protection. The same $A_{2b}$ receptors are critical for postconditioning's protection. Thus, adenosine is both an important trigger and a mediator of cardioprotection (Cohen and Downey 2008).

Results of a prospective, randomized, placebo-controlled trial of intracoronary adenosine administration performed during percutaneous coronary intervention has

shown that this procedure improved the angiographic and electrocardiographic results in patients with acute myocardial infarction with ST-segment elevation (Grygier et al. 2011).

### Preconditioning the Myocardium

Conditioning of myocardial cells to an oxidative stress prior to IRI may limit the consequences of this injury. Preconditioning the myocardium with HBO before reperfusion has been shown to have a myocardial protective effect by limiting the infarct size post-ischemia and reperfusion. Current evidence suggests that HBO preconditioning may partly attenuate IRI by stimulating the endogenous production of nitric oxide (NO), which has the ability to reduce neutrophil sequestration, adhesion, and associated injury, and improve blood flow (Yogaratnam et al. 2008), HBO preconditioning induced NO may play a role in providing myocardial protection during operations that involve an inevitable episode of IRI and protection of the myocardium from the effects of IRI during cardiac surgery.

## *Beta Blockers as Cardioprotectives*

Beta blockers are versatile therapeutic agents used for the treatment of several disorders. They have been widely used in the treatment of uncomplicated hypertension and are still recommended as first-line agents in national and international guidelines. They have been used to treat ischemic heart disease, due to negative chronotropic and inotropic properties, thus inducing a decrease in myocardial consumption of oxygen and nutrients, allowing a better balance between nutritional needs and the supply provided by the compromised coronary blood flow. Their cardioprotective efficacy, however, is based on extrapolation into primary prevention of data relative to the reduction of mortality observed in the 1970s in patients with previous MIs. Critical reanalysis of older trials, together with several meta-analyses, has shown that in patients with uncomplicated hypertension, beta blockers do not have any protective effect with regard to coronary artery disease. The newer beta blockers, such as nebivolol and carvedilol, which show vasodilatory properties and a more favorable hemodynamic and metabolic profile, may be more effective in reducing morbidity and mortality, but this remains to be proven (De Caterina and Leone 2010).

Carvedilol, a nonselective beta-blocker with alfa-blocker properties, is currently used to treat hypertension, heart failure and coronary artery disease. It has a unique mitochondrial-related mechanism of cardioprotection. Antioxidant properties of carvedilol can be used to minimize oxidative stress, a powerful inducer of mitochondrial permeability transition. Carvedilol has a positive impact of on the prognosis of patients with coronary heart disease (Carreira et al. 2006).

Nebivolol is a selective β1 receptor blocker with nitric oxide-potentiating vasodilatory effect used for the treatment of hypertension as well as left ventricular

failure. It avoids many of the side effects of other beta blockers that are caused by their blockade of β2 receptors. Its cardioprotective action has been demonstrated againt chemotherapy-induced cardiotoxicity.

## *Cardioprotective Effects of Growth Hormone*

Cardioprotective effects of growth hormone (GH)/insulin-like growth factor 1(IGF-1) axis remains controversial, and the underlying mechanism(s) for such actions are unclear. Growth hormone-releasing hormone (GHRH) has been postulated to directly activate cellular reparative mechanisms within the injured heart, in a GH/IGF-1 independent fashion. A study on rat model of myocardial infarction (MI) has shown that GHRH agonists can activate cardiac repair after MI, suggesting the existence of a potential signaling pathway based on GHRH in the heart (Kanashiro-Takeuchi et al. 2010). The phenotypic profile of the response to a potent GHRH agonist has therapeutic implications.

## *Cardioprotection by Blocking Complement Activation*

As mechanisms of cardiac damage are better understood and various involved pathways are being studied, new strategies are being developed including those directed at inflammation and immune disturbances. Since the complement system is a central mediator of inflammation, it is recognized as a promising therapeutic target. An anti-C5 monoclonal antibody was developed to block the final stage of complement activation. Pexelizumab (Alexion Pharmaceuticals) is a single chain, short-acting anti-C5 antibody and is used for reperfusion after myocardial infarction, or for CABG surgery with cardiopulmonary bypass (Patel and Ghatak 2008). A proprietary molecule, rC3-1 (InCode BioPharmaceutics Inc), which targets complement protein C3, is in development for control of complement-mediated disturbances such as inflammation that occur in various diseases including myocardial infarction. The molecule has been shown to be suitable for controlled delivery and self-administration via subcutaneous injection or inhalation.

## *Cardioprotection by Resveratrol*

Red wine contains many compounds that may have therapeutic use, including resveratrol (3,4′,5-trihydroxytrans-stilbene). Since resveratrol could be administered both in the diet and as a therapeutic agent, defining appropriate concentrations requires understanding of the pharmacokinetics. Resveratrol absorption is rapid but plasma concentrations are low as it is rapidly and efficiently converted into relatively hydrophilic phase-2 conjugates and metabolites, which are then rapidly

excreted via the urine and bile. Resveratrol is an effective antioxidant in vivo by increasing NO synthesis and also maintaining the reduced intracellular redox state via the thioredoxin system. Further, activation of sirtuins (one class of lysine deacetylases) may mediate the cardiovascular responses shown by resveratrol. Studies on animal models of human disease suggest that resveratrol has the potential to decrease cardiovascular symptoms in patients with myocardial infarction, arrhythmias, hypertension, cardiomyopathies, fibrosis, atherosclerosis, thrombosis, and diabetes, but, as yet, human clinical trials are scarce. Cardioprotection by resveratrol in rodent models may rely on mechanisms producing pharmacological preconditioning in the heart including reducing reactive oxygen species, improving vasorelaxation and angiogenesis, preventing inflammation and apoptosis, delaying atherosclerosis as well as decreasing cardiovascular remodeling. Interventional studies in humans need to be completed before resveratrol can be considered as a standard therapeutic agent. Therefore, future studies should focus on obtaining the level of evidence required to determine whether resveratrol can be added to the list of evidence-based therapies for cardiovascular diseases that includes renin-angiotensin system inhibitors, beta-adrenoceptor antagonists, and calcium entry blockers (Kroon et al. 2010).

## *HDL-Mediated Pharmaceutical Cardioprotection*

Increasing attention has focused on the role of high-density lipoprotein function as a target for cardioprotection. Apolipoprotein A-IMilano (AIM) involves a single amino acid mutation of the major wild-type protein carried on high-density lipoprotein (HDL) particles. Early evidence of beneficial activities of AIM has stimulated support in its development as a potential therapy to reduce cardiovascular risk.

ETC-216 represents a lipid-deplete form of HDL containing recombinant AIM. While early evidence suggests that administration of ETC-216 promotes rapid regression of coronary atherosclerosis, bringing this compound to clinical practice will require further trials that evaluate its impact on cardiovascular events (Nicholls et al. 2011).

## *Nicorandil for Cardioprotection*

Nicorandil in Angina (IONA) clinical trial demonstrated that the use of nicorandil, a $K_{ATP}$ channel opener with anti-anginal effect, reduced future cardiovascular events in patients with stable angina. To determine the potential stabilizing effect of nicorandil on stabilization of coronary atherosclerotic plaques, preintervention intravascular ultrasound-virtual histology was performed prospectively in patients with stable angina pectoris (Izumiya et al. 2011). The nicorandil group demonstrated a larger percentage of fibrous tissue and a smaller percentage of necrotic core tissue compared with the non-nicorandil group. The effect of nicorandil on atherosclerotic

lesion formation was also studied in a mouse model of atherosclerosis. Lipid profiles were unaffected, but the area of atherosclerotic lesion and plaque necrosis were significantly reduced following 8 weeks of nicorandil treatment in ApoE-deficient mice fed an atherogenic diet. Nicorandil significantly reduced the expression levels of endoplasmic reticulum stress biomarkers, C/EBP homologous protein (CHOP) and glucose regulated protein/BiP in atherosclerotic lesions. Nicorandil significantly attenuated tunicamycin-induced CHOP upregulation in cultured THP-1 macrophages. The conclusion from these studies was that nicorandil exerts its anti-atherogenic effect by mechanisms different from those of statins. Long-term nicorandil treatment can be considered as a second-line prevention therapy for patients with coronary artery disease.

## *Statins for Cardioprotection in Dilated Cardiac Myopathy*

Statins are generally used for lowering cholesterol and indirectly protect against coronary heart disease due to hypercholesterolemia. Statins also have an immunomodulating effect in inflammatory processes affecting the heart. Dilated cardiomyopathy (DCM) is a multifactorial disease in which there is enlargement and systolic dysfunction of one or both ventricles leading to congestive heart failure (CHF), which is a progressive disease with high morbidity and mortality, suggesting that important pathogenic mechanisms are not modified by current symptomatic treatment. Inflammation plays a role in the development and progression of CHF, influencing heart contractility and hypertrophy, promoting apoptosis, and contributing to myocardial remodeling. Immunomodulating treatments, such as statins, have shown promising results in patients with cardiomyopathies. Statins, by inhibiting inflammation and reducing endothelial dysfunction, lead to the improvement of left ventricular function and exercise tolerance in patients with DCM in New York Heart Association class II or III and with normal or increased levels of lipids (Bielecka-Dabrowa et al. 2011).

## *Role of Proteomics in Cardioprotection*

### Proteomics of Cardioprotective Role of Preconditioning

Combined proteomic and metabolomic analyses provide direct evidence of the effect of protein expression on cellular processes, and this approach has been used to investigate the cardioprotective mechanisms following exposure to nitrate, which is an important bioactive molecule, capable of conferring cardioprotection and a variety of other benefits in the cardiovascular system (Perlman et al. 2009). Nitrate administration resulted in a short-lived increase in cardiac nitrate levels, but substantial elevations in cardiac ascorbate oxidation. This was accompanied by significant improvements in cardiac contractile recovery following ischemia-reperfusion after

preconditioning with low or high nitrate doses. There was significant nitrite-induced protein modifications (including phosphorylation) revealed by MS-based proteomic studies. Altered proteins included those involved in metabolism (e.g., aldehyde dehydrogenase 2), redox regulation (e.g., protein disulfide isomerase A3), contractile function (e.g., filamin-C), and serine/threonine kinase signaling (e.g., protein kinase A R1$\alpha$). Thus, brief elevations in plasma nitrite trigger a concerted cardioprotective response characterized by persistent changes in cardiac metabolism, redox stress, and alterations in myocardial signaling. These findings help elucidate possible mechanisms of nitrite-induced cardioprotection and have implications for nitrite dosing in therapeutic regimens. A similar mechanism may underpin the cardioprotective value of physical exercise and a diet containing nitrite/nitrate-rich foods.

#### Cardioprotection Based on Study of Cardiac Mitochondrial Proteome

Mitochondria can represent a threat to the cell under hypoxic conditions because they can generate reactive oxygen species. However, cardiomyocytes are equipped with an oxygen-sensing pathway that involves prolyl hydroxylase oxygen sensors and hypoxia-inducible factors, which induces a tightly regulated program to keep ischemic mitochondrial activity under control (Cadenas et al. 2010).

Proteomic methods are useful in studying the cardiac mitochondrial proteome, which are important, particularly those associated with cell death and protection. Proteomic data from these studies have contributed to addressing the role of mitochondria in cardioprotection (Gucek and Murphy 2010).

## *Protection of the Blood Vessels*

Apart from the protection of the myocardium, protection of the coronary arteries as well as other arteries in the human body is an important consideration in management of cardiovascular diseases. Two important processes that require protective strategies are atherosclerosis of the arterial wall and prevention of proliferation of endothelium following injury or therapeutic interventions such as angioplasty. The latter will be discussed in more detail in Chap. 9.

An example of the application of molecular biology to this problem is the study of Toll-like receptors (TLRs). The capacity of about 10 TLRs to recognize conserved patterns on many bacterial and viral pathogens is remarkable, but TLRs, in particular to TLR2 and TLR4, have been assigned detrimental roles in immune and cardiovascular disease. Using human and murine systems, a study has investigated the consequence of TLR3 signaling in vascular disease (Cole et al. 2011). The responses of human atheroma-derived smooth muscle cells (AthSMC) and control aortic smooth muscle cells (AoSMC) were compared to various TLR ligands. AthSMC exhibited a specific increase in TLR3 expression and TLR3-dependent functional responses. Exposure to dsRNA in vitro and in vivo induced increased

expression of both pro- and anti-inflammatory genes in vascular cells and tissues. An unexpected finding was that neointima formation in a perivascular collar-induced injury model was reduced by the systemic administration of the dsRNA analog Poly(I:C) in a TLR3-dependent manner. Furthermore, genetic deletion of TLR3 dramatically enhanced the development of elastic lamina damage after collar-induced injury. Deficiency of TLR3 accelerated the onset of atherosclerosis in hypercholesterolemic ApoE−/− mice. These data indicate a protective role for TLR signaling in the vessel wall.

## Important Advances in Cardiovascular Therapeutics

The most important advances in cardiovascular therapeutics are:

1. Advances in cardiovascular surgery
2. Cell therapy: transplantation of cells for repair of myocardial damage
3. Gene therapy: to promote angiogenesis for repair of heart
4. Statin drugs to reduce hypercholesterolemia
5. Platelet blocker drugs to prevent blood clots
6. Nitric oxide therapeutics
7. Stents, particularly drug-eluting stents, for preventing restenosis of coronary arteries
8. Advances in cardiac catheterization
9. Cardiovascular imaging for interventional cardiology and cardiac diagnosis
10. Advances in cardiac monitoring and pacemakers

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# Chapter 2
# Drug Delivery to the Cardiovascular System

## Introduction

Delivery of therapeutics to the human body is an important part of treatment of various diseases. Biotechnology plays an important role in improving therapeutic delivery. Drug delivery is discussed in a book on this topic (Jain 2008). Drug delivery to the cardiovascular system is the topic of a special report (Jain 2011). This chapter deals mostly with delivery of drugs for cardiovascular diseases and biological therapies such as cell and gene therapies are described in separate chapters.

## Routes of Drug Delivery to the Cardiovascular System

Various routes of drug delivery used for treatment of cardiovascular disorders are shown in Table 2.1.

## Local Administration of Drugs to the Cardiovascular System

### *Intramyocardial Drug Delivery*

Direct intramyocardial injection may permit local delivery of protein and gene therapy agents for myocardial and coronary artery disease. Little is known about the immediate fate of materials administered via percutaneous endomyocardial catheters or via surgical epicardial injection. Animal experimental studies have shown that despite direct intramyocardial administration, a significant fraction of injected material is not retained locally. Catheter-based needle endomyocardial injection is associated with equivalent or superior retention of injected material compared with open chest epicardial injection. Loss may involve a combination of channel leakage, venous, and lymphatic return.

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**Table 2.1** Routes of drug delivery used for treatment of cardiovascular disorders

| |
|---|
| *Systemic administration of drugs by various routes (non-targeted)* |
| Oral |
| Intramuscular |
| Intravenous |
| Transdermal |
| Sublingual |
| Inhalation |
| *Systemic administration for targeted effect* |
| *Local administration* |
| Drug delivery into the myocardium: direct intramyocardial injection, drug-eluting implanted devices |
| Drug delivery via coronary venous system |
| Injection into coronary arteries via cardiac catheter |
| Intrapericardial drug delivery |
| Release of drugs into arterial lumen from drug-eluting stents |

Local intramyocardial delivery (IMD) is under active clinical investigation for cell therapies to treat congestive heart failure (CHF), and gene therapies to induce revascularization of ischemic myocardium in coronary artery disease. Locally delivered agents can migrate away from the site of delivery through pathways that include lymphatics. Postdelivery redistribution can be observed using fluorescent tracers of different physical geometries. This approach provides a means to characterize these pathways and to delineate their importance in local cardiovascular drug delivery. Animal experimental studies show that IMD may provide a means to administer hydrophilic agents to the periadventitial zone of the arterial wall to limit restenosis (Altman et al. 2003). The lack of redistribution of the 15 μm microspheres supports the potential for cells to remain localized to the site of administration for an extended period of time.

Erythropoietin (EPO) protects myocardium against ischemic injury, but it also produces polycythemia, which can cause thromboembolic complications. Following induction of myocardial infarction in rats, local sustained delivery by intramyocardial injection of EPO combined with hydrogel has been shown to enhance the cardioprotective effect without causing polycythemia (Wang et al. 2009). The hydrogel allows sustained release of EPO, which inhibits cell apoptosis and increases angiogenesis that subsequently reduces infarct size and improves cardiac function without apparent adverse effects.

## *Drug Delivery via Coronary Venous System*

Retrograde coronary venous perfusion can preserve myocardium during experimental coronary artery occlusion and has been used clinically to deliver oxygenated

blood to ischemic myocardium during unstable angina or high-risk percutaneous transluminal coronary angioplasty (PTCA). Retrograde delivery of drugs accelerates coronary thrombolysis, preserves regional myocardial function, and limits infarct size in animal models. Modifications of the coronary venous retroperfusion technique have allowed access to smaller coronary venous branches and minimization of systemic effects of local drug delivery. Retrograde coronary sinus perfusion as an adjunct in high-risk coronary PTCA is limited by its inability to provide systemic hemodynamic support during circulatory collapse. Targeted and specific gene delivery to myocardium with transfection rates superior to intra-arterial or systemic injection may be a promising new application for this technique.

### *Intrapericardial Drug Delivery*

Pharmacologic modulation of the contents of the pericardial space has been shown to influence the response of coronary arteries to balloon injury. Local delivery of various drugs into the lumen of coronary arteries has been limited by short residence time, as well as by highly variable concentration of the deposited agent. Compounds introduced into the pericardial fluid can access coronary arteries with intramural concentrations, which typically vary by 10- to 15-fold, while endoluminal delivery results in a remarkably wide intramural concentration range with up to 33,000-fold variability. Compounds placed into the pericardial space penetrate into coronary tissue with greater consistency than seen after endoluminal delivery and provide for prolonged coronary exposure to agents. Intrapericardial (IPC) delivery appears to offer substantial advantages over endoluminal administration in the coronary arteries with respect to residence time and reproducibility. This approach has not been developed for clinical use.

Amiodarone and sotalol are frequently used in the treatment of atrial fibrillation, but oral and intravenous (IV) therapy with these drugs has suboptimal efficacy and is associated with serious extracardiac side effects. In experimental animals, IPC delivery produces steeply decreasing drug concentrations from epicardium to endocardium in both atria and ventricles with plasma drug concentrations that are significantly lower in IPC than in IV treated animals (Bolderman et al. 2009). IPC delivery of amiodarone and sotalol increases atrial drug concentration and antiarrhythmic effects at reduced plasma drug concentrations. These potential benefits are particularly prominent for IPC delivered amiodarone.

## Formulations for Drug Delivery to the Cardiovascular System

Various formulations for drug delivery to the cardiovascular system are shown in Table 2.2.

**Table 2.2** Formulations for drug delivery to the cardiovascular system

| |
|---|
| *Sustained release preparations* |
| *Controlled release preparations* |
| *Preparations for targeted delivery* |
| *Special preparations* |
| Proteins and peptides |
| Microparticles and nanoparticles |
| Liposomes |
| *Cell and gene therapies* |

## *Sustained and Controlled Release*

These two formulations are used often in cardiology and will be defined. Sustained release (SR) preparations are not new but several new modifications are being introduced. They are also referred to as "long acting" or "delayed release" as compared to "rapid" or "conventional" release preparations. The term sometimes overlaps with "controlled release," which implies more sophisticated control of release and not just confined to the time dimension. Controlled release implies consistency but release of drug in SR preparations may not be consistent. The rationale of developing SR is:

- To extend the duration of action of the drug
- To reduce the frequency of dosing
- To minimize the fluctuations in plasma level
- Improved drug utilization
- Less adverse effects

Limitations of SR products are:

- Increase of drug cost
- Variation in the drug level profile with food intake and from one subject to another
- The optimal release form is not always defined and multiplicity of SR forms may confuse the physician as well as the patient

SR is achieved by either chemical modification of the drug or modifying the delivery system, for example, use of a special coating to delay diffusion of the drug from the system. Chemical modification of drugs may alter such properties as distribution, pharmacokinetics, solubility, or antigenicity. One example of this is attachment of polymers to the drugs to lengthen their lifetime by preventing cells and enzymes from attacking the drug.

Controlled release implies regulation of the delivery of a drug usually by a device. The control is aimed at delivering the drug at a specific rate for a definite period of time independent of the local environments. The periods of delivery are

usually much longer than in case of SR and vary from days to years. Controlled release may also incorporate methods to promote localization of drug at an active site. Site-specific and targeted delivery systems are the descriptive terms used to denote this type of control.

### Programming the Release at a Defined Time

Approaches used for achieving programmed or pulsatile release may be physical mechanisms such as swelling with bursting or chemical actions such as enzymatic degradation. Capsules have been designed that burst after a predetermined exposure to an aqueous environment. Physical factors that can be controlled are the radius of the sphere, osmotic pressure of the contents and wall thickness, as well as elasticity. Various pulsatile release methods for oral drug delivery include the Port system (a semipermeable capsule containing an osmotic charge and an insoluble plug) and Chronset System (an osmotically active compartment in a semipermeable cap. Other systems have impermeable capsules and erodible plugs, which are formed either by direct compression of various hydrophilic polymers, such as hydroxypropyl methylcellulose and propylene oxide, or by solidification of a meltable material directly into the capsule orifice.

### Dosage Formulation of Calcium Channel Blockers

Although effective as antihypertensive agents, recent studies have suggested that calcium channel blockers (CCBs) may be detrimental and may promote adverse cardiovascular events. It is the rate of drug delivery into the systemic circulation that produces profound effects on the hemodynamic and neurohumoral responses to a dihydropyridine CCB drug. During chronic treatment with dihydropyridines, major fluctuations in blood pressure (rapid onset and offset of antihypertensive effects) during the dosing interval may persist for drugs and formulations that are short acting. In contrast, slow-release formulations of otherwise rapidly absorbed dihydropyridines achieve a more gradual and sustained antihypertensive effect. It is probable that newer CCB formulations that do not provoke intermittent sympathetic activation and do not evoke a cardioacceleratory response would be expected not to promote adverse cardiovascular events. Comparison of sustained and controlled release preparations of verapamil will be described as an example.

### Sustained and Controlled Release Verapamil

With the immediate release formulation, more than 90% of the orally administered dose of verapamil is absorbed. Because of rapid biotransformation of verapamil during its first pass through the portal circulation, bioavailability ranges from 20% to 35%. Peak plasma concentrations are reached between 1 and 2 h after oral administration. Chronic oral administration of 120 mg of verapamil every 6 h resulted in plasma levels of verapamil ranging from 125 to 400 ng/mL, with higher

values reported occasionally. A nonlinear correlation between the verapamil dose administered and verapamil plasma levels does exist.

The dose-ranging population pharmacokinetics of an osmotic controlled release verapamil has been studied in healthy subjects and patients with angina or hypertension. Immediate release verapamil has an approximate fourfold greater apparent clearance than controlled release verapamil in both healthy volunteers and patients. Waxy microparticles prepared by the ultrasonic spray congealing technique are promising solvent-free devices for controlling the release of verapamil in vivo.

## *Methods of Administration of Proteins and Peptides*

Several of the current drugs are proteins or peptides and many more in this category are in development. Various possible routes for administration of proteins and peptides are:

- Parenteral
- Transdermal
- Inhalation
- Transnasal
- Oral
- Rectal
- Implants
- Cell and gene therapies
- Use of special formulations

Injection still remains the most common method for administration of proteins and peptides. Efforts are being made to use needle-free or painless injections and also to improve the controlled delivery by parenteral route. The ideal delivery system for proteins and peptides should have the following characteristics:

- It should be patient friendly
- Noninvasive
- It should provide good stability of the product
- Bioavailability should be high
- Dosing and absorption rates should be reproducible
- Should be cost-effective

### Delivery of Peptides by Subcutaneous Injection

Subcutaneous still remains predictable and controllable route of delivery for peptides and macromolecules. However, there is need for greater convenience and lower cost for prolonged and repeated delivery. An example of refinement of subcutaneous delivery is MEDIPAD (Elan Pharmaceutical Technologies), which is

a combination of "patch" concept and a sophisticated miniaturized pump operated by gas generation.

### Depot Formulations and Implants

These are usually administered by injection and must ensure protein/peptide stability. One of the formulations used is poly(lactide-co-glycide) sustained release. Implants involve invasive administration and also must ensure protein/peptide stability. Implantable titanium systems provide drug release driven by osmotic pumps. This technology has been extended to other proteins such as growth hormone. Nutropin Depot (Genentech/Alkermes) is the first long-acting form of growth hormone that encapsulates the drug in biodegradable microspheres that release the hormone slowly after injection. It reduces the frequency of injection in children with growth hormone deficiency from once daily to once a month.

### Poly(Ethylene Glycol) Technology

Poly(ethylene glycol) or PEG, a water soluble polymer, is a well-recognized treatment for constipation. When covalently linked to proteins, PEG alters their properties in ways that extend their potential uses. Chemical modification of proteins and other bioactive molecules with PEG – a process referred to as PEGylation can be used to tailor molecular properties to particular applications, eliminating disadvantageous properties or conferring new molecular functions. This approach can be used to improve delivery of proteins and peptides. Advantages of PEG technology are:

- Increase of drug solubility
- Increase of drug stability
- Reduction of immunogenicity
- Increase in circulation lifetime
- Improvement of release profile

Enzyme deficiencies for which therapy with the native enzyme is inefficient (due to rapid clearance and/or immunological reactions) can now be treated with equivalent PEG-enzymes.

### Microencapsulation for Protein Delivery

Microencapsulation of recombinant cells is a novel and potentially cost-effective method of heterologous protein delivery. A "universal" cell line, genetically modified to secrete any desired protein, is immunologically protected from tissue rejection by enclosure in microcapsules. The microcapsule can then be implanted in different recipients to deliver recombinant proteins in vivo.

### Localized Delivery of Biomaterials for Tissue Engineering

Several biomaterials have been developed for cell-based tissue replacement strategies. Biodegradable polymer scaffolds can be used as space-filling matrices for tissue development and barriers to migration of epithelial cells in tissue conductive approaches. Inductive approaches involve sustained delivery of bioactive factors, such as protein growth factors and DNA, to alter cell function in localized regions. Factors can be released from highly porous polymer scaffolds for tissue development. Cell-based approaches involve seeding of cells onto polymeric scaffolds in vitro and subsequent transplantation of the scaffold. New scaffold materials are being developed that address specific tissue engineering design requirements, and in some cases attempt to mimic natural extracellular matrices. These strategies together offer the possibility of predictably forming specific tissue structures, and may provide solutions to problems such as periodontal defects and alveolar bone resorption.

### Oral Delivery of Proteins and Peptides

This is the most desirable route. Orally ingested proteins are rapidly converted to constituent amino acids before absorption. The aim is absorption of proteins and peptides in an intact state. Three main obstacles to oral delivery of proteins are:

1. Destruction of proteins by acid and proteolytic enzymes in the stomach
2. Difficulties of transporting molecules across the epithelial layer lining the intestine
3. Bioavailability is low

The oral delivery of proteins is challenging and has often been considered an unattainable goal; nevertheless, the efforts to develop oral formulations of proteins continue to be made. The approaches devised to overcome the obstacles for the absorption of proteins from the gastrointestinal tract are various, and include the coadministration of enzyme inhibitors and/or absorption enhancers, the encapsulation in liposomes, microemulsions, or biodegradable particles, and the modification of proteins through the attachment of chemical moieties. Efforts for oral protein drug delivery are directed along the following lines:

*Inhibition of the proteolytic enzymes in the small intestine*. This is achieved by a variety of enzymatic inhibitors such as soy bean trypsin inhibitor, aprotinin, and encapsulation in lipid emulsion.

*Passage across the intestinal epithelium*. This is facilitated by transcellular and paracellular mechanisms. The former have not proven efficient to date and paracellular methods are being pursued as they are more effective but these involve damage to the epithelial cells. There is also the choice between small intestine and the large intestine (colon) as the site of absorption. The upper small intestine has a large absorptive area with a dynamic absorption process. However, the environment is hostile and would denature most proteins. The colon has the advantage of a less

hostile environment with longer emptying time. The disadvantages are a small absorptive area, a passive absorptive process and difficulty in ensuring that the drug will be released at the appropriate site. The rectum offers another opportunity where some of the disadvantages of colon can be eliminated but this route is not popular with most patients.

*Formulation of proteins/peptides with protective carriers*. Although this is the most attractive route, there are difficulties in transporting molecules across mucosal epithelia and the bioavailability is still low. Oral delivery of peptides and proteins can be carried with hydrophilic drugs dissolved in oily formulations.

*Covalent addition of polymers to the protein or peptide*. Protein or peptide can be chemically changed by addition of polymers composed of water- and fat-soluble elements.

*Protein/peptide unfolding*. A few companies are developing these techniques. One of the promising technologies in this category is gastrointestinal mucoadhesive patch system (GI-MAPS™) from BioSerenTach Co Ltd, which (1) protects the drug from the hydrolysis action of digestive enzymes by preventing the permeation of GI fluid inside the system with water-insoluble polymer membrane and (2) obtains high concentration gradient of both drug and absorption enhancer on the intestinal mucosal surface by adhering to the target site of the intestine. After oral administration of gelatin capsule containing GI-MAPS, drugs in the formulation are protected from the gastric juice in the stomach by enteric film on the adhesive layer (adhesion site-controlling layer) and protection layer. When GI-MAPS is transferred to the small intestine, the adhesion site-controlling layer of GI-MAPS is dissolved at the target site of the small intestine, and then GI-MAPS adhere to the intestinal mucosal membrane. As a result of adhesion, the drug carrying layer of GI-MAPS existing between protecting layer and adhesive layer forms a closed space. Drug in the closed space is protected from the attack of the digestive enzymes in the intestinal lumen. When drug in the drug carrying layer is dissolved by intestinal fluid, high concentration of drug solution can be obtained. As a result of high concentration gradient of drug between the system and the enterocytes, formulated drug can be efficiently absorbed. In addition, when an absorption enhancer coexists with a drug in the drug carrying layer, the concentration of enhancer as well as drug in this closed space reaches to high degree. Under this condition, optimal absorption enhancing effect can be obtained. This technology can be applied for the delivery of interferon, insulin, calcitonin, granulocyte colony-stimulating factor (G-CSF), epoietin, growth hormone, interleukins, desmopressin, etc.

*Peptide transporters*. PEPT1 and PEPT2 play an important role in the maintenance of protein nutrition and in the handling of endogenously derived peptides. These transporters also have significant pharmacological and pharmacokinetic relevance to the transport of various peptide-like drugs.

*Fusion with transferrin*. Transferrin is a plasma protein found in blood. Humans naturally produce transferrin to move iron through the blood to the liver, spleen,

and bone marrow. Transferrin can be fused with large, protein-based drugs such as G-CSF to create a new oral compound that is capable of surviving the journey through the gastrointestinal tract and then able to cross over into the bloodstream to be used by the body. Transferrin-based recombinant fusion protein technology represents a promising approach for future development of orally effective peptide and protein drugs.

## Targeted Drug Delivery to the Cardiovascular System

Cell-selective targeted drug delivery has emerged as one of the most significant areas of biomedical engineering research, to optimize the therapeutic efficacy of a drug by strictly localizing its pharmacological activity to a pathophysiologically relevant tissue system. The approach for targeting therapeutic agents to a specific pathological site is to exploit biologically germane ligand-receptor interactions prevailing at the tissue site. Delivery devices chemically modified by such ligands can home-in on specific cell receptors and deliver active therapeutics to the pathological site without adversely affecting the surrounding normal tissue. Various approaches to deliver therapeutic substances to the cardiovascular system are shown in Table 2.3.

### *Immunotargeting of Liposomes to Activated Vascular Endothelial Cells*

This is a strategy for site-selective delivery in the cardiovascular system. Endothelial-selective delivery of therapeutic agents, such as drugs or genes, would provide a useful tool for modifying vascular function in various disease states. A potential molecular target for such delivery is E-selectin, an endothelial-specific cell surface molecule expressed at sites of activation in vivo and inducible in cultured human umbilical vein endothelial cells (HUVEC) by treatment with cytokines such as recombinant human interleukin 1beta (IL-1beta). Liposomes of various types (classical, sterically stabilized, cationic, pH-sensitive), each conjugated with MAb H18/7, a murine monoclonal antibody that recognizes the extracellular domain of E-selectin, bound selectively and specifically to IL-1beta-activated HUVEC at levels up to 275-fold higher than to inactivated HUVEC. E-selectin-targeted immunoliposomes appeared in acidic, perinuclear vesicles 2–4 h after binding to the cell surface, consistent with internalization via the endosome/lysosome pathway. Activated HUVEC incubated with E-selectin-targeted immunoliposomes, loaded with the cytotoxic agent doxorubicin, exhibited significantly decreased cell survival, whereas inactivated HUVEC were unaffected by such treatment. These results demonstrate the feasibility of exploiting cell surface activation markers for the endothelial-selective delivery of biologically active agents via immunoliposomes. Application of this targeting approach in vivo may lead to novel therapeutic strategies in the treatment of cardiovascular disease.

**Table 2.3** Targeted delivery of therapeutic substances to the cardiovascular system

| Approach | Application |
|---|---|
| Targeted thrombolysis by intra-arterial catheter delivering thrombolytic substances such as urokinase to avoid the systemic complications | Vascular thrombotic diseases |
| Catheter-based coronary drug delivery | Controlled release delivery of antithrombotic drugs, genes, and antisense agents to inhibit endothelial proliferation |
| Stents releasing heparin | Prevention of restenosis after angioplasty |
| Perivascular administration of bFGF to promote angiogenesis and revascularization while avoiding the systemic effects of bFGF administration | Myocardial ischemic disease |
| Immunotargeting of liposomes to activated vascular endothelial cells | Endothelial-selective delivery of therapeutic agents, such as drugs or genes. Provides a useful tool for modifying vascular function in various cardiac diseases |
| Transvenous placement of defibrillator catheters ionophoretic implants that can release drugs in an adjustable and reliable manner through the application of an electric current | Delivery of antiarrhythmic agents such as lidocaine, ibutelide, and propanolol for treatment of cardiac arrhythmias |
| Epicardial placement of cyclosporine-containing collagen around the transplanted heart to prevent rejection without systemic effect of the drug | Cardiac transplantation |
| Cell therapy by implantation of myocytes or intravenous injection of hematopoietic stem cells | To regenerate and replace the damaged myocardium |
| Gene therapy to insert genes that would prevent atherosclerosis, induce vascularization, stop apoptosis and promote the regeneration of new cells or to inhibit abnormal proliferation of endothelial cells after angioplasty | Myocardial ischemia<br>Peripheral vascular disease<br>Prevention of restenosis after angioplasty |
| Antisense therapy | Prevention of restenosis after angioplasty<br>Hypertension |

*bFGF* basic fibroblast growth factor

## *PEGylated Biodegradable Particles Targeted to Inflamed Endothelium*

Drug carriers made from biodegradable polymers [e.g., poly(lactic acid), PLA] are easily prepared, have a long shelf life, can carry several orders of magnitude more drug than MAbs, and can be designed to have well-defined drug-release rates. Because of these attributes, it is well accepted that biodegradable drug carriers complement and expand the possibilities of targeted drug delivery afforded by

other carriers (e.g., liposomes and MAbs). Targeted, PEGylated biodegradable particles have been developed with adhesive properties similar to that of leukocytes. The targeted particles exhibit; (i) significant (up to 15-fold) selective adhesion to inflamed endothelium, relative to noninflamed endothelium; (ii) significant (sixfold) selective adhesion for cytokine inflamed endothelium, relative to non-cytokine-treated endothelium, in vivo; and (iii) significant (up to tenfold) enhancement in adhesion to trauma-induced inflamed endothelium, in vivo, due to the addition of a targeting ligand (Sakhalkar et al. 2003). The particles can be made to target endothelial cell adhesion molecules known to play a role in leukocyte recruitment including vascular cell adhesion molecule (VCAM)-1, E-selectin, P-selectin, and intercellular adhesion molecule (ICAM)-1, which are increased at sites of pathological inflammation. Thus, the fields of drug delivery and vascular cell–cell adhesion physiology have been linked to successfully develop PEGylated biodegradable polymeric particles that exhibit selective adhesion to inflamed endothelium at levels similar to that of leukocytes. The role of endothelium in vascular diseases continues to be explored, and opportunities for targeted drug delivery via the endothelium will further increase.

## Devices for Cardiovascular Drug Delivery

Historically, cardiovascular system therapy has used many devices ranging from vascular catheters to electronic pacemakers and artificial implants. Some of these devices are tied in with pharmacotherapy and are relevant to drug delivery. A classification of devices used in relation to drug delivery to the cardiovascular system is shown in Table 2.4.

**Table 2.4** Classification of devices for drug delivery to the cardiovascular system

| |
|---|
| *Special injection devices* |
| Perivascular injections of drugs: e.g., microneedle to deliver drug around the coronary artery |
| *Intra-arterial catheters* |
| For placement of other devices such as stents (see Chap. 9) |
| For delivery of drugs to various targets in the cardiovascular system |
| For delivery of embolic materials to close arteriovenous fistulas |
| *Devices for arrhythmia linked to pharmacotherapy: hybrid drug-device* |
| Combining drug therapy with catheter ablation |
| Combining drug therapy with pacemakers |
| Combining drug therapy with cardioverter defibrillators |
| *Implants for reconstruction or functional replacement of cardiovascular components* |
| Combination with drugs to prevent thrombosis |
| *Nanotechnology-based devices* |
| Growth factors encapsulated in nanoparticles in scaffolds for tissue regeneration |
| *Devices for restenosis: e.g., drug eluting stents* |

## *Local Drug Delivery by Catheters*

Local endovascular delivery (LED) of therapeutics by a catheter is mostly for peripheral vascular disease. A variety of agents including pharmaceuticals, cells, and genes are used. Indications include the following:

- Reduction of restenosis after balloon angioplasty or stenting (see Chap. 9)
- Prevention of intimal hyperplasia/stenosis in vein bypass grafts
- Promotion of revascularization in limb ischemia by promoting angiogenesis development
- Prevention of thrombus formation
- Stabilization of therosclerotic plaques to reduce lesion progression and diminish the risk of their rupture
- Catheter-directed thrombolysis in deep vein thrombosis (DVT)

Pulmonary embolism is a well-known complication of DVT. Patients with acute DVT are usually treated by anticoagulation, which prevents the propagation of the existing clot but does not dissolve the thrombus. Despite receiving anticoagulation therapy, a significant number of patients, particularly those with iliofemoral DVT, develop the post-thrombotic syndrome (PTS), which is manifested by leg pain, swelling, skin discoloration, and venous claudication; venous ulceration is the most severe form of PTS. DVT may eventually recanalize but can produce venous insufficiency and/or reflux because of damage to the venous valves. Catheter-directed venous thrombolysis has been proposed as a means of reducing the risk of PTS, as this actually dissolves the acute thrombus, restores venous patency, and restores venous valve function (Wicky 2009).

The ideal characteristics for clinical application of LED are as follows although none of the available devices meets all of the criteria.

- Minimal reactivity between the catheter and the agent being delivered
- Delivery of a high percentage of therapeutic agents to the vessel wall
- Minimal downstream escape
- Atherosclerotic accumulation following delivery
- An easy procedure with a short completion time

Types of catheters used for LED include passive diffusion catheters, pressure-driven balloon catheters and mechanically assisted injection catheters.

*Passive diffusion catheters*. Balloon catheters that facilitate passive diffusion of the pharmacological agent to reach only the innermost layers of the artery include double, channel, microporous, and hydrogel-coated balloons (angioplasty balloon catheters whose exterior is coated with an adsorbent polymer that has been soaked with a chosen drug).

*Pressure-driven balloon catheters.* These devices include the circumferential needle injection balloon catheter and the porous balloon catheter. The perforated

balloon catheter is designed to facilitate intramural drug delivery. These catheters have small holes between 50 and 90 mm in diameter that permit fluid infusion by direct contact with the artery wall.

*Mechanically assisted injection catheters*. Iontophoretic catheters enhance the penetration across the endothelial cell lining by generating an electrical current gradient to drive charged or hydrophilic molecules into the adventitial layer of the artery wall.

Biocompatibility of the catheter and the therapeutic agent is important. Passage of cells or other biological agents through an injection catheter exposes them to shearing forces, pressure, and the surface material of the inner lumen. These forces can rupture cells, or break DNA strands. Factors most likely to reduce this damage include the speed of injection, composition of the suspension fluid as well as the stability and concentration of the cells and DNA.

## *Micro-Infusion Catheters for Periarterial Injection*

Mercator MedSystems has created the first micromedical device to inject safely through vessel walls. Using standard interventional procedures, physicians can position Mercator's Bullfrog®Micro-Infusion catheter in peripheral artery (Fig. 2.1)


**Fig. 2.1** Bullfrog® Micro-Infusion catheter for peripheral artery injection. Illustrations courtesy of Mercator MedSystems


**Fig. 2.2** Cricket® Micro-Infusion catheter for coronary artery injection. A single injection to the outside of the vessel results in liquid compounds diffusing around the vessel (circumferentially), up and down the vessel (longitudinally), and inward through the vessel layers (transmurally). The microscopic needle puncture is so small that it heals almost instantly. Illustrations courtesy of Mercator MedSystems

or the Cricket® Micro-Infusion catheter the coronary artery (Fig. 2.2). The Micro-Infusion Catheter is marked with radio-opaque labels for fluoroscopic visualization. While the Micro-Infusion catheter is closed, the microneedle is hidden and does not injure vessel walls as it is maneuvered into place. When the Micro-Infusion is opened, the microneedle slides through the vessel wall to inject drugs directly to the surrounding tissue. The drugs are deposited around the outside of the vessel and diffuse inward through the vessel layers. The microscopic puncture is so small that it heals almost immediately, limiting trauma and bleeding.

The differences between perivascular (outside the vessel) and intravascular (inside the vessel) drug delivery include:

- Perivascular delivery allows drug retention at the treatment site versus being washed away in the bloodstream.
- Perivascular delivery is immediately visualized with minute amounts of contrast medium, proving positively that the drug is localized at the target.
- Perivascular delivery allows direct treatment vessel wall and surrounding tissue, which in case of coronary arteries is the myocardium.

- A single injection can diffuse to bathe several centimeters of tissue or vessel – circumferentially, longitudinally, and transmurally.
- Fatty tissue outside the vessel retains most injected therapeutic substances as a natural depot for extended treatment.

Using the Micro-Infusion in coronary arteries results in high concentrations of drug at the injection site and low levels of the drug dispersed throughout the coronary arterial tree. When drugs are delivered to the adventitia, affinity between the drug and the tissue can create a local depot around the artery. This provides a sustained source of diffusion. This system has undergone preclinical testing for delivery of:

- Drugs. Delivery of drugs via the Micro-Infusion should have a strong impact on preventing restenosis following angioplasty and stenting. Further studies are required to understand the complementary effect of delivering low levels of anti-inflammatory compounds to the coronary tree for the treatment of unstable plaque.
- Stem cells. Micro-Infusion can be used for the delivery of a patient's own adult stem cells to the heart directly through the coronary arteries.
- Proteins
- Genes
- Polymer beads up to 15 μm in diameter

**Automated Drug Delivery System for Cardiac Failure**

Pharmacological support with inotropes and vasodilators to control decompensated hemodynamics requires strict monitoring of patient condition and frequent adjustments of drug infusion rates, which is difficult and time consuming. Previous systems attempted to directly control systemic arterial pressure and systemic arterial pressure by estimating their responses to drug infusions. This approach is inapplicable because of the difficulties in simultaneous estimating responses to the infusion of multiple drugs.

A novel automated drug delivery system has been devised for simultaneous control of systemic arterial pressure (AP), cardiac output (CO), and left atrial pressure (P(LA)) in acute heart failure (Uemura et al. 2010). The circulatory equilibrium framework established by the authors previously directly controls pumping ability of the left heart with dobutamine, stressed blood volume with dextran/furosemide, and arterial resistance with nitroprusside. It showed that AP, CO, and P(LA) are determined by equilibrium of the mechanical properties of the circulation, that is, pumping ability of the left heart, stressed blood volume, and systemic arterial resistance. The current system directly controls the three mechanical properties with cardiovascular drugs including inotropes and vasodilators, thereby controlling AP, CO, and P(LA). Furthermore, by precisely controlling bradycardia and LV inotropy, this system enables improvement of cardiac energetic efficiency while preserving AP, CO, and P(LA) within acceptable ranges. In conclusion, by directly controlling the mechanical properties of the heart and vessel, the automated system achieves comprehensive management of hemodynamics in acute heart failure.

## *DDS in the Management of Ischemic Heart Disease*

Current treatment for ischemic heart disease with pharmaceuticals and revascularization procedures is directed at relief of symptoms and does not treat the underlying pathophysiologic process. Major approaches to this problem are lysis of the thrombus, surgical endarterectomy, dilatation of the stenosed arteries by percutaneous angioplasty or bypass revascularization procedures. Cell therapy could repair the myocardial damage following coronary artery occlusion. Gene therapy strategies could reduce the incidence of heart disease by correcting the gene defects responsible for insulin-dependent diabetes mellitus and hyperlipidemia and hypertension. Methods of drug delivery for ischemic heart disease are shown in Table 2.5.

The blood vessels are lined with a monolayer of endothelial cells which maintain a crucial role in maintaining blood fluidity and vascular patency. Endothelial cells generate a number of molecules that protect blood vessels from injury and major disease processes such as thrombosis and atherosclerosis. The three best known of these are prostacyclin, tissue plasminogen activator (tPA), and nitric oxide (NO). Several of these active molecules have been purified or synthesized and administered systematically for the treatment of arterial thrombotic disorders. For example, intravenous infusion of tPA has been shown to reduce the morbidity and mortality of coronary heart disease. The disadvantages of this compound are:

- Bioavailability is limited by neutralization of tPA by plasminogen activator inhibitor.
- Adverse effects such as hemorrhage ensue when large doses are given.
- It acts selectively at fibrin-generating sites and systemic administration often does not produce adequate local concentration.

**Table 2.5** Drug delivery in ischemic heart disease

| |
|---|
| *Oral administration of sustained and controlled release drugs* |
| *Intramuscular injections* |
| *Intravenous administration of novel therapies* |
| Thrombolysis |
| Emulsified formulations of halogenated anesthetics as cardioprotective agents |
| *Intraarterial* |
| Thrombolysis |
| Local delivery of drugs the site of lesion |
| *Injections into the heart* |
| *Transdermal* |
| *Nitric oxide-based therapies* |
| *Nanoparticle-based drug delivery to the cardiovascular system* |
| *Cell therapy* |
| *Gene therapy* |
| *RNAi* |

### Intravenous Emulsified Formulations of Halogenated Anesthetics

Preconditioning against myocardial infarction by inhalation of volatile anesthetics is well known. New emulsified formulations of halogenated anesthetics administered intravenously reduce myocardial infarct size when administered before prolonged ischemia and reperfusion in experimental animals. Lipid vehicle in the formulation produces transient increases in heart rate, whereas emulsified volatile anesthetics had no effect on hemodynamics before coronary occlusion. Emulsified isoflurane, enflurane, and sevoflurane reduce infarct size of the area at risk. Administration of lipid vehicle or emulsified sevoflurane does not produce sedation or respiratory depression in conscious rabbits. It is concluded that intravenous emulsified halogenated anesthetics produce acute and delayed preconditioning against myocardial infarction.

### Injectable Peptide Nanofibers for Myocardial Ischemia

Endothelial cells can protect cardiomyocytes from injury through platelet-derived growth factor (PDGF)-BB signaling. PDGF-BB induces cardiomyocyte Akt phosphorylation in a time- and dose-dependent manner and prevents apoptosis via PI3K/Akt signaling. An experimental study in rats using injectable self-assembling peptide nanofibers, which bound PDGF-BB in vitro, demonstrated sustained delivery of PDGF-BB to the myocardium at the injected sites for 14 days (Hsieh et al. 2006). This blinded and randomized rat study showed that injecting nanofibers with PDGF-BB, but not nanofibers or PDGF-BB alone, decreased cardiomyocyte death and preserved systolic function after myocardial infarction. A separate blinded and randomized study showed that PDGF-BB delivered with nanofibers decreased infarct size after ischemia/reperfusion. PDGF-BB with nanofibers induced PDGFR-β and Akt phosphorylation in cardiomyocytes in vivo. These data demonstrate that PDGF-BB signaling and the in vitro finding can be translated into an effective in vivo method of protecting myocardium after infarction. Furthermore, this study shows that injectable nanofibers allow precise and sustained delivery of proteins to the myocardium with potential therapeutic benefits.

### Delivery of Angiogenesis-Inducing Agents for Myocardial Ischemia

Angiogenesis therapy is aimed at revascularization of the myocardium in ischemic heart disease. Surgical procedures involve implantation of blood vessels. Medical therapy is mainly by use of growth factors such as VEGF, FGF, and placental growth factor (PIGF). These are proteins given by injection. Growth factors delivered by gene therapy include VEGF, FGF-4, HGF, Del-1, and HIF-1. Cell therapy for myocardial ischemia involves implantation of various types of cells including hematopoietic stem cells that are engineered to produce angiogeneic agents. Routes of administration include intravenous injection, intracoronary infusion, and percutaneous intramyocardial delivery.

Delivery of the therapeutic agent at the desired site for certain duration of time is essential for efficacy of angiogenesis-inducing agents. Although strong preclinical data and small human trials have supported the concept of angiogenic revascularization, so far the double blind randomized placebo-controlled trials have failed to conclusively demonstrate a clinically meaningful benefit. Further studies are required to determine the optimal dose, formulation, route of administration, and combinations of growth factors and the utility of adjunctive endothelial progenitor cell or other stem cell supplementation, to provide safe and effective therapeutic myocardial neovascularization.

## *Drug Delivery for Cardiac Rhythm Disorders*

Cells are delivered in combination with devices for control of cardiac rhythm disorders. This section will describe an example of drug delivery with a device for postoperative atrial fibrillation (AF), which is a complication associated with approximately 500,000 surgeries performed each year: 30% of the coronary artery bypass grafting and 60% of the cardiac valve replacement.

CardioPolymers Inc's postoperative AF product, Plexisyl-AF, developed in collaboration with clinicians at the Cleveland Clinic Foundation in the USA and Imperial College School of Medicine (London, UK), employs a nonablative, nonsurgical approach whereby a biopolymer that prevents the development of persistent atrial fibrillation is prophylactically injected into a specific location of a patient's heart prior to the conclusion of an open chest cardiothoracic procedure. The AF treatment "Kit" includes three proprietary components; a hemostatic sealant derived from human plasma, a disposable two-channel applicator to facilitate delivery of the biopolymer, and a disposable hand-held electrical stimulation device used to accurately detect the precise location of the injection. The administration of the biologic material into the epicardial fat pads achieves temporary modulation of the autonomic nervous system of the heart by blocking the vagal inputs to the heart that can serve as an initiating trigger for atrial fibrillation. There are no known pharmacological, immunological, or metabolic interactions that are believed to occur, and the Company's preclinical studies have demonstrated that the biological material dissipated in less than 30 days after introduction into the heart. This procedure has been tested in clinical trials.

## *Sustained and Controlled-Release Nitrate for Angina Pectoris*

If nitrates are administered around the clock, tolerance to their effects develops rapidly. Once tolerance has developed, the drug is probably not having any beneficial effect. The drug must be taken in such a manner that therapeutic serum concentrations are only present for 12–14 h of the day. Nitrates have many alternative

methods of delivery. Isosorbide nitrate (ISDN), the common nitrate preparation, is used as sustained release preparations, ointment, and transdermal patches. ISDN works directly on the vascular smooth muscle in an identical manner to endogenous nitric oxide. ISDN has a short half-life and is primarily effective clinically by metabolism to the active metabolite isosorbide-5-mononitrate, which has an average half-life of 5 h. This is the active ingredient in the two new nitrate preparations Imdur and Ismo. These two preparations are no more effective than ISDN and the serum concentrations of isosorbide-5-mononitrate are similar for the three preparations. The duration of ISDN's antianginal effect is approximately 6 h, which is similar to most sustained release preparations. The alternate forms of delivery of ISDN have not been shown to be more effective than oral ISDN. The ointment and patches offer a longer duration of action, but this has little advantage given the necessity for an 8–10 h drug-free period to prevent tolerance.

### Transdermal Nitrate Therapy

Traditionally, sublingual nitroglycerin has been used for the prevention of angina pectoris in coronary artery heart disease. Following oral administration, nitroglycerin undergoes intense gastric hydrolysis, and is mostly destroyed by the first-pass effect. The development of an attenuation phenomenon of the therapeutic effect has been frequently reported during continuous treatments at fixed doses. Transdermal administration overcomes some of these drawbacks of systemic administration of nitroglycerin. However, continuous treatment with transdermal nitroglycerin leads to tolerance development within the first day of application. Effective long-term therapy can be provided by interval treatment with nightly patch removal, but even during the hours of intermittent patch application there is rapid attenuation of initial effects. Interval therapy with increasing nitroglycerin concentrations provides unattenuated anti-ischemic and antianginal efficacy during the hours of treatment and circumvention of early tolerance during chronic application. This modified pharmacokinetic profile can be regarded as a model for an improved dosage regimen in nitrate interval therapy. Supplemental L-arginine prevents the development of tolerance during continuous transdermal nitroglycerin therapy. Besides nitroglycerin, testosterone in hypogonad males and estrogens in menopausal women frequently relieve angina pectoris.

Various preparations of transdermal nitroglycerin are available and some are in development. These include the following:

- Deponit-Nitroglycerin (Schwarz Pharma/Labtec)
- Epinitril (Rottapharm)
- Minitran Transdermal (3 M Pharmaceuticals)
- NITRODUR (Schering-Plough)
- Nitroglycerin Patch (Stowic)
- Transderm-Nitro (ALZA)
- Trinipatch (Sanofi-Aventis)

The NITRO-DUR Transdermal Infusion System (Schering-Plough) is a flat unit designed to provide continuous controlled release of nitroglycerin through intact skin. The rate of release of nitroglycerin is linearly dependent upon the area of the applied system. Steady-state plasma concentrations of nitroglycerin are reached by about 2 h after application of a patch and are maintained for the duration of wearing the system. Upon removal of the patch, the plasma concentration declines with a half-life of about an hour. Minitran Transdermal (3 M Pharmaceuticals) is also a 24 h transdermal nitroglycerin delivery system for prevention of angina pectoris due to coronary artery disease. The onset of action of transdermal nitroglycerin is not sufficiently rapid for this product to be useful in aborting an acute attack.

Trinipatch (Sanofi-Aventis) is a small, transparent, matrix, monolayer nitroglycerin patch with an absorption promoter. Its systemic and local safety and clinical efficacy have been studied in clinical trials. The systemic safety, assessed by adverse event reporting, was good. A very good stability of cardiovascular parameters was observed, with no reflex tachycardia. The local safety, evaluated by the Draize scale, was also satisfactory. A significant reduction of the number of angina attacks and the numbers of doses of nitroglycerin was observed. The percentage of pain-free patients increased from 18.2% at the start of the study to 76% at the end of the trial. Trinipatch 5 mg is marketed now.

A new glyceryl trinitrate transdermal patch Epinitril (Rottapharm) contains the drug uniformly dissolved in a monolayer pressure-sensitive acrylates vinyl acetate copolymer adhesive matrix. The patch provides an intense flux rate of glyceryl trinitrate through the skin. For its small size, thinness, flexibility, transparency, easiness of application and of removal, and for its good tolerability, Epinitril is very patient friendly, a quality that improves the compliance with the long-term therapeutic courses needed in angina pectoris.

## *Vaccines Delivery for Hypertension*

Hypertension, which is one of the most common diseases of humans, is associated to increased morbidity, mortality, and cost to society. However, hypertension is the most common reversible risk factor for cardiovascular diseases. The renin-angiotensin system (RAS) commands an important role in the regulation of blood pressure, and so, at present, has been a target for clinical control by drugs acting on the system. Despite the fact that effective drugs are available, blood pressure is successfully controlled in only one third of the patients. Undesirable side effects and the poor compliance with oral drug therapy are blamed for the poor results. Immunization against renin and the angiotensins has been attempted in the past but the results have not been satisfactory. Immunization against angiotensin-I with PMD-3117 vaccine, angiotensin-II with CYT006-AngQb vaccine, and targeting of angiotensin-II type 1A receptor with ATR12181 vaccine have provided optimism in the development of a hypertension vaccine (Pandey et al. 2009). Vaccines could induce

long-lasting effects with a dosing interval of months, increasing patient acceptability and compliance, and thus a better control of high blood pressure.

CYT-006-AngQb, under development by Cytos Biotechnology AG, is a vaccine in which a peptide derived from the angiotensin II molecule is conjugated to the surface of the highly repetitive structure of virus-like particles. In spontaneously hypertensive rat models, CYT-006-AngQb induced strong angiotensin-II-specific antibodies and reduced systolic blood pressure. In a phase I clinical trial, single doses of CYT-006-AngQb were well tolerated in healthy males. In a phase II trial, multiple doses of CYT-006-AngQb administered to patients with mild-to-moderate hypertension reduced blood pressure; the average half-life was longer than all currently available oral hypertension medications (Phisitkul 2009). There were no significant side effects except for local skin reactions at the injection site. Given the novel mechanism of CYT-006-AngQb, and the potential to complement other hypertension treatments, success in ongoing phase II trials in patients with hypertension would potentially make this therapy a valuable addition to the therapeutic armamentarium for hypertension.

## *Drug Delivery in the Management of Pulmonary Hypertension*

Pulmonary arterial hypertension (PAH) is a disease of the pulmonary vasculature but the sequelae are cardiovascular. The resulting increase in the right ventricular after load leads to right ventricular failure and death. The treatment options are limited, expensive, and associated with significant side effects. Inhaled NO is used to treat various cardiopulmonary disorders associated with pulmonary hypertension. The rationale is based on the fact that NO, given by inhalation, only dilates those pulmonary vessels that perfuse well-ventilated lung units. As a result, pulmonary gas exchange is improved while pulmonary vascular resistance is reduced and pulmonary blood flow is increased. Premature newborns and those with pneumonia or heart problems often develop neonatal PAH, a NO-deficient state characterized by pulmonary vasoconstriction, and systemic hypoxemia. Inhaled NO has been successfully applied to treat persistent PAH of the newborn, reducing the need for extracorporeal life support. Apart from inhaled NO, novel therapies including inhaled prostacyclin and other pulmonary vasodilators such as sildenafil are under investigation. Gene transfer to restore the deficiency of NOS gene may alleviate pulmonary hypertension.

### Prostacyclin by Inhalation

Iloprost (Actelion's Ventavis®) is an inhaled synthetic analog of prostacyclin (PGI2) that produces potent pulmonary vasodilation and inhibits platelet aggregation, among other benefits. Prostacyclin has multiple physiological actions, including vasodilation, inhibition of platelet aggregation, antiproliferative as well as anti-inflammatory effects, and enhanced cardiac contractility.

Iloprost is indicated for the treatment of patients with PAH (WHO Group 1) with NHYA Class III or IV symptoms. It significantly increases patient improvement after 12 weeks of treatment compared to baseline on a composite endpoint of improved exercise capacity 30 min after dosing, improvement of at least one NHYA class and no clinical deterioration. It significantly improves 6 min walk distance at week 12 with a 10% or greater increase in individual walk distance. Although inhaled iloprost has been approved for the treatment of adults with PAH, a study showed that it caused sustained functional improvement in some children with PAH, although it occasionally induced bronchoconstriction (Ivy et al. 2008). Most patients tolerated the transition from intravenous to inhaled prostanoid therapy.

### Endothelin Receptor Antagonist Treatment of PAH

Bosentan (Actelion's Tracleer®), the first oral dual endothelin receptor antagonist, is approved for the treatment of PAH and made available by subsidiaries in the USA, the EU, Japan, Australia, Canada, Switzerland, and other markets worldwide. Tracleer® has a label extension in the USA for the treatment of patients with mildly symptomatic WHO Functional Class II PAH. Results of a randomized study demonstrate that the addition of inhaled iloprost in patients with PAH with reduced exercise capacity on bosentan monotherapy is safe and efficacious (McLaughlin et al. 2006).

## *Anticoagulation in Cardiovascular Disease*

Anticoagulation is used for the prophylaxis and treatment of vascular thrombosis. Many cardiovascular disorders are characterized by thromboembolism. Deep venous thrombosis may occur from prolonged immobilization or inactivity. Heparin, one of the most potent anticoagulants widely used for the treatment and prevention of deep vein thrombosis and pulmonary embolism, is currently available to patients only by parental administration.

### Oral Heparin

Currently, the most common indication for heparin therapy is the prevention of blood clot formation following major surgical procedures lasting longer than 30 min, such as angioplasty or heart surgery. Emisphere is developing an oral formulation of heparin. Unfractionated heparin and warfarin have been the most commonly prescribed anticoagulant agents to prevent thrombus formation. Generally, physicians prefer heparin over warfarin because heparin has a rapid onset of anticoagulant activity, a short physiological half-life and significantly fewer incidences of drug–drug interactions. These pharmacological properties facilitate dose adjustment and contribute to heparin's relatively large margin of safety.

A proposed new oral delivery system of heparin conjugates it with deoxycholic acid (DOCA), which is reformulated by adding dimethyl sulfoxide to increase its bioavailability. The chemical conjugate of heparin and DOCA in the soluble state could be efficiently absorbed in the intestine. Therefore, this system has been proposed as a new strategy of oral heparin delivery for the treatment of patients who are at high risk to deep vein thrombosis or pulmonary embolism (Kim et al. 2005).

Another study was carried out to design and evaluate a highly efficient stomach-targeted oral delivery system for low molecular weight heparin (LMWH). In an in vivo study in rats, 6 kDa LMWH (300 IU) formulation displayed a relative bioavailability of 10.7% in contrast to the control displaying a relative bioavailability of 2.1% (Schmitz et al. 2005). These results suggest that mucoadhesive thiolated polymers are a promising tool for the noninvasive stomach-targeted systemic delivery of LMWH as model for a hydrophilic macromolecular polysaccharide.

### Low Molecular Weight Heparin–Loaded Polymeric Nanoparticles

Low molecular weight heparin (LMWH) nanoparticles have been prepared as potential oral heparin carriers. The nanoparticles are formulated using an ultrasound probe by water-in-oil-in-water emulsification and solvent evaporation with polymers. The mean diameter of LMWH–loaded nanoparticles ranges from 240 to 490 nm and is dependent on the reduced viscosity of the polymeric organic solution. The highest encapsulation efficiencies are observed when Eudragit polymers are used in the composition of the polymeric matrix. The in vitro LMWH release in phosphate buffer from all formulations ranges from 10% to 25% and is more important (two- to threefold) when esterase is added into the dissolution medium. The in vitro biological activity of released LMWH, determined by the anti-factor Xa activity with a chromogenic substrate, is preserved after the encapsulation process, making these NP good candidates for oral administration.

### Transdermal Anticoagulants

Heparin and low-molecular weight heparin are the most commonly used anticoagulants and are administered by intravenous or subcutaneous injections. However, injections of heparin have the potential risk of bleeding complications and the requirement of close monitoring in some cases. Transdermal drug delivery offers an attractive alternative to injections due to minimization of pain, sustained release of drugs, control of the rate of administration, and termination on demand. However, the dose of transdermally delivered heparin is limited by low skin permeability. Low-frequency ultrasound increases permeability of pigskin in vitro and rat skin in vivo and allows delivery of biologically active doses of heparin and low-molecular weight heparin transdermally. Prolonged contact of transdermally delivered heparin with pigskin reduces the biologic activity of heparin, although no such deactivation is observed during short exposures. Transdermally delivered low-molecular weight heparin results in sustained aXa levels in the blood – an index of biological activity. This result is in strong contrast to subcutaneous or intravenous injections of low-molecular

weight heparin, which resulted in only temporary elevations of aXa level. It is concluded that transdermal delivery of low-molecular weight heparin is a potential alternative to injections.

## *Thrombolysis for Cardiovascular Disorders*

Acute myocardial ischemia due to coronary artery occlusion or acute ischemic stroke due to occlusion of cerebral arteries are indications for thrombolytic therapy with agents such as alteplase (Genentech Inc's Activase), an approved tissue plasminogen activator. Catheter-directed thrombolysis for the treatment of deep venous thrombosis is safe and effective, regardless of the agent used. Alteplase thrombolysis has shown to be effective for emergency treatment of pulmonary embolism when given as a bolus dose. Alteplase is given intravenously but intra-arterial therapy has also been investigated. Pharmacomechanical thrombolysis is also under investigation by use of rheolysis and ultrasound.

### Use of Ultrasound to Facilitate Thrombolysis

Intravenous alteplase (recombinant tissue plasminogen activator) has been shown to be beneficial within a short 3 h window after stroke. Ultrasound has a thrombolytic capacity that can be used for pure mechanical thrombolysis or improvement of enzyme-mediated thrombolysis. Mechanical thrombolysis with ultrasound needs high intensities at the clot that may have unwanted side effects, whereas improvement of enzymatic thrombolysis can be done at the safer energy levels used in diagnostic ultrasound. Methods of improving enzymatic thrombolysis with ultrasound include intra-arterial delivery of thrombolytic agents with an ultrasound-emitting catheter and targeted and non-targeted noninvasive transcranial ultrasound delivery during intravenous thrombolytic infusion. Animal and clinical studies of sonothrombolysis have shown clot lysis, and accelerated recanalization of arterial occlusion has been seen in in vitro flow models, occluded peripheral and coronary arteries, and intracerebral arteries. Controlled clinical trials to test safety management and effectiveness of both strategies are in progress.

### Delivery of Alteplase Through the AngioJet Rheolytic Catheter

The AngioJet Xpeedior rheolytic system (MEDRAD Interventional/Possis) is an endovascular thrombectomy catheter that employs heparinized saline jets to create a low-pressure zone around the catheter tip, causing a vacuum (Bernoulli/Venturi) effect. It consists of three main components: (1) a drive unit, (2) the pump set that contains the piston chamber, and (3) the rheolytic catheter. Thrombus is drawn into the catheter, where it is fragmented by shear forces from the jets and removed from the vessel into a collection reservoir. Enzymatic proteins are susceptible to fragmentation and inactivation from the hydraulic shear stress found in systems using jet or ultrasonic nebulization or aerosolization.

**Table 2.6** Drug delivery for peripheral arterial disorders

| |
|---|
| Oral sustained and controlled release preparations |
| Delivery of thrombolytic agent to the clot through an arterial catheter |
| Delivery of growth factors to promote angiogenesis in ischemic limbs |
| Drug-eluting stents |
| Transdermal |
| Immune modulation therapy |
| Nitric oxide-based therapy |
| Antisense therapy |
| Cell therapy |
| Gene therapy |

Alteplase solutions have been analyzed following delivery through the AngioJet Xpeedior rheolytic thrombectomy device to characterize the viability of proteins exposed to high shear stress (Semba et al. 2005). No significant fragmentation or aggregation of protein was observed. Alteplase solutions, when delivered through the AngioJet Xpeedior rheolytic thrombectomy device, remain stable and biologically active in vitro. This method needs to be tested clinically.

## *Drug Delivery for Peripheral Arterial Disease*

Various methods for drug delivery for peripheral arterial disorders (PADs) are listed in Table 2.6. Cholesterol-lowering and antihypertensive therapies also improve PAD.

### Delivery of Thrombolytic Agent to the Clot Through a Catheter

Alfimeprase (ARCA) is an enzyme produced by recombinant DNA technology. It is a thrombolytic agent that is intended to directly degrade fibrin when delivered through an arterial catheter to the site of a blood clot. It directly degrades fibrin, a protein that provides the scaffolding for blood clots. In clinical studies, this direct mechanism of action has been shown to provide rapid clot dissolution with a well-tolerated safety profile. Alfimeprase's thrombolytic activity is localized to the site of delivery because it is rapidly inactivated by α-2 macroglobulin, a naturally occurring protein in the blood, as it moves away from the site of delivery and into the general blood circulation. This clearance mechanism focuses the thrombolytic activity to the site of the clot and offers the potential to minimize bleeding side effects. It is in phase III clinical trials for the treatment of peripheral arterial occlusion.

### Delivery of Growth Factors to Promote Angiogenesis in Ischemic Limbs

Therapeutic vascularization remains a significant challenge in regenerative medicine applications, where the goal is to induce vascular growth in ischemic tissue or scale up tissue-engineered constructs. Engineered PEG-based bioartificial hydrogel

matrices presenting protease-degradable sites, cell-adhesion motifs, and growth factors have been shown to induce the growth of vasculature in vivo (Phelps et al. 2010). Compared to injection of soluble VEGF, these matrices can deliver sustained in vivo levels of VEGF over 2 weeks as the matrix degrades. When implanted subcutaneously in rats, degradable constructs containing VEGF and arginine-glycine-aspartic acid tripeptide induce a significant number of vessels to grow into the implant at 2 weeks with increasing vessel density at 4 weeks. The mechanism of enhanced vascularization is likely cell-demanded release of VEGF, as the hydrogels may degrade substantially within 2 weeks. In a mouse model of hind limb ischemia, delivery of these matrices produced significantly increased rate of reperfusion. These results support the application of engineered bioartificial matrices for delivery of vascular growth factors to promote vascularization in regenerative therapies for peripheral ischemic disease.

### Immune Modulation Therapy for Peripheral Arterial Disease

Vasogen's Celacade™ technology involves the ex vivo exposure of a sample of autologous blood to three oxidative stress factors (heat, an oxidative environment, and ultraviolet light), followed by intramuscular reinjection. A double-blind, placebo-controlled pilot study has assessed the effect of Celacade™ technology on skin blood flow in patients with symptomatic peripheral arterial occlusive disease (Edvinsson et al. 2003). No significant differences were detected between various groups for resting or peak postischemic laser Doppler fluxmetry values for dorsal foot skin blood flow. Patients randomized to Celacade™ experienced a progressive decrease in the time to peak postischemic skin blood flow, reaching statistical significance at 2 months. Treated patients experienced a 26.1 s decrease in time to peak blood flow compared to a 7.9 s decrease in the placebo group. Similar but less striking results were achieved for $tcpO_2$ recovery time to 50% of pre-ischemia values (treated group, $p = 0.035$; placebo group, $p = \text{ns}$). The improved recovery rates of postischemic dorsal foot skin blood flow in a group of patients with moderately advanced peripheral arterial disease was probably due to improved endothelial function.

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# Chapter 3
# Role of Nitric Oxide in Cardiovascular Disorders

## Introduction

Although one of the simplest biological molecules in nature, nitric oxide (NO) has found its way into nearly every phase of biology and medicine, ranging from its role as a critical endogenous regulator of blood flow and thrombosis to a principal neurotransmitter mediating erectile function as well as a major pathophysiological mediator of inflammation and host defense. Role of NO in medicine and pharmacology is discussed in more detail in a special report on this topic (Jain 2011b).

These major discoveries have stimulated intense and extensive research into a vast array of fields including chemistry, molecular biology, and gene therapy. NO has so many facets that it is uniting many fields of medicine including neurology, cardiology, and immunology. As a reactive gas, NO functions both as a signaling molecule in both endothelial and nerve cells, as well as a killer molecule by activated immune cells.

NO, like other small neutral gases, can diffuse across cell to its site of action. Because it contains an unpaired electron, it is extremely active. The major mechanism of termination of biological action of NO is its reaction with $O_2$ to form $NO_2$, which in aqueous solution results in the formation first of $N_2O_3$ and then $N_2O_2^-$. $N_2O_2^-$ is then further oxidized by a variety of endogenous oxidants to $NO_3^-$, which is relatively innocuous. Free NO is a transient species with a half-life of only about 5 s. Hence, most studies on NO actions are based on the activity of nitric oxide synthase (NOS). NO binds to the heme moiety of guanylate cyclase, and causes greater than 400-fold activation of the enzyme.

Endothelial NO production results in local formation of adducts that may act as storage forms of NO. Experimental studies indicate that NO photolytically released in the tissue originates from species with photophysical properties similar to those reported for low-molecular-weight S-nitrosothiols, as well as from nitrite. The relative contribution of these potential NO stores to the extent of photorelaxation (ability to release NO when illuminated with light and subsequently relax vascular smooth muscle) is consistent with their concentrations detected biochemically in vascular tissue when their photoactivity was taken into account. These observations indicate that intravascular nitroso species and nitrite both have the potential to

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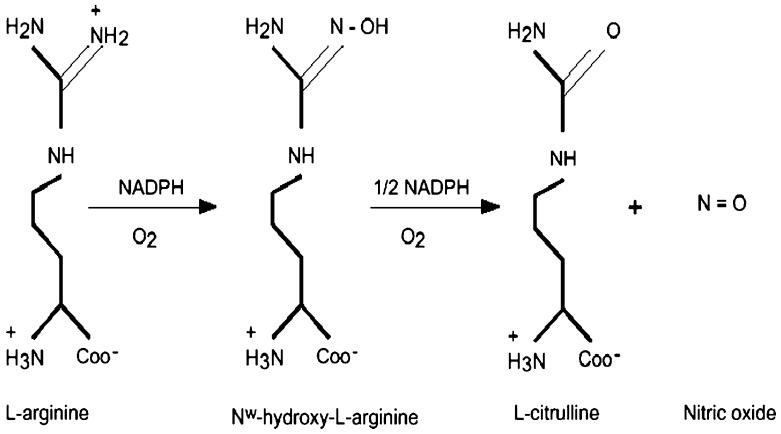


**Fig. 3.1** Biosynthesis of nitric oxide (NO). L-arginine is converted to NO in two successive steps of which a two-electron oxidation of L-arginine to N-w-hydroxy-L-argininine is the first, then converted to NO and citrulline, utilizing one and half NADPH and $O_2$. Both steps require $Ca^{2+}$ and calmodulin as activators and are accelerated by tetrahydrobiopterin (©Jain PharmaBiotech)

release physiologically relevant quantities of NO independent of enzymatic control by NO synthase.

Classically, NO was administered as a chemically synthesized donor such as nitroglycerine but many other methods of administration are available now. There are two major sources of nitrate and nitrite in the body: the endogenous L-arginine-NO synthase pathway (NOS) and the diet.

NO is generated by NOS. L-arginine is converted to L-citrulline and NO in a two-step reduction with five electrons donated by NADPH (see Fig. 3.1).

NOS is of three different types, each with different tissue distributions and encoded by different genes. These are sometimes referred to by numbers but identification of location is less likely to cause an error.

1. Neuronal nitric oxide synthase (nNOS) regulated by $Ca^{2+}$ through an association with calmodulin.
2. Inducible nitric oxide synthase (iNOS) is contained in macrophages and is not regulated posttranslationally.
3. Endothelium nitric oxide synthase (eNOS) is similar to nNOS but is membrane bound.

Cloning of the isoforms of NOS has revealed that they share only approximately 50% of primary sequence homology, suggesting that they may differ from each other in regulatory aspects. The term cNOS is used for constitutively expressed NOS (eNOS and nNOS) as distinguished from the inducible form (iNOS).

## *Role of NO in Physiology of the Cardiovascular System*

Endothelial production of NO has become a major research area in vascular biology. NO mediates multiple physiological and pathophysiological processes in the cardiovascular system. NO may trigger short- and long-term effects, which are either beneficial or deleterious, depending on the molecular targets with which it interacts. These interactions are governed by local factors (like the redox state). In the cardiovascular system, the major targets involve not only guanylyl cyclase, but also other heme proteins, protein thiols, iron-nonheme complexes, and superoxide anion (forming peroxynitrite). The latter has several intracellular targets and may be cytotoxic, despite the existence of endogenous defense mechanisms. These interactions may either trigger NO effects or represent releasable NO stores, able to buffer NO and prolong its effects in blood vessels and in the heart. Some of the most important effects that NO exerts in the vascular wall are potentially vasoprotective, because these effects maintain important physiological functions such as vasodilation, anticoagulation, leukocyte adhesion, smooth muscle proliferation, and the antioxidative capacity.

The redox siblings nitroxyl (HNO) and NO have often been assumed to undergo casual redox reactions in biological systems. However, several studies have demonstrated distinct pharmacological effects for donors of these two species. In one study, infusion of the HNO donor Angeli's salt into normal dogs resulted in elevated plasma levels of calcitonin gene-related peptide, whereas neither the NO donor diethylamine/NONOate nor the nitrovasodilator nitroglycerin had an appreciable effect on basal levels (Miranda et al. 2003). Conversely, plasma cGMP was increased by infusion of diethylamine/NONOate or nitroglycerin but was unaffected by Angeli's salt. These results suggest the existence of two mutually exclusive response pathways that involve stimulated release of discrete signaling agents from HNO and NO. Calcitonin gene-related peptide release is suggested to occur via altered calcium channel function through binding of HNO to a ferric or thiol site. The orthogonality of HNO and NO may be due to differential reactivity toward metals and thiols, and in the cardiovascular system may ultimately be driven by respective alteration of cAMP and cGMP levels.

### NO and Atrial Natriuretic Peptide

Atrial natriuretic peptide (ANP) is part of an endocrine system that maintains fluid and pressure homeostasis by modulating cardiac and renal function. The physiologic functions of the ANP in healthy humans and in patients with cardiovascular disease are not fully understood. From a physiological standpoint, the most important factor governing ANP secretion is mechanical stretching of the atria, which normally occurs when extracellular fluid volume or blood volume is elevated. In addition, the ability of several vasoconstrictors to increase ANP secretion can be traced to their indirect effects on atrial stretch via increases in cardiac preload or afterload. Whether vasoconstrictors such as angiotensin II and vasopressin have a direct positive or negative effect on ANP secretion has not been determined with

certainty. Endothelin, a potent vasoconstrictor, stimulates ANP secretion and augments stretch-induced ANP secretion. Increase in ANP release produced by cardiac ischemia is partly mediated by endothelin. NO, produced by endothelial cells, also inhibits ANP secretion acting through cyclic GMP as an intracellular messenger. ANP levels are elevated in patients with congestive heart failure (CHF) and other cardiac diseases; measurement of ANP may be used in to aid diagnosis and prognosis. In addition, synthetic ANPs such as nesiritide are available for use in management of patients with acutely decompensated CHF.

### NOS in the Cardiac Myocyte

Cardiac myocytes contain two constitutive NOS isoforms with distinct spatial locations, which allows for isoform-specific regulation. One regulatory mechanism for NOS is substrate (L-arginine) bioavailability. Mitochondrial Arg II negatively regulates nNOS activity, most likely by limiting substrate availability in its microdomain (Steppan et al. 2006). These findings have implications for therapy in pathophysiologic states such as aging and heart failure in which myocardial NO signaling is disrupted. At the level of intracardiac ganglia and myocytes, microdomains of NOS isoforms are found in the vicinity of their targets (Fig. 3.2).

nNOS localized to the sarcoplasmic reticulum can regulate $Ca^{2+}$ fluxes enhanced by β-adrenoceptor activation and myocardial excitability in the cardiac ventricle. Although the main functional outcome of NO signaling in parasympathetic control of cardiac rate appears to work through nNOS-dependent presynaptic pathways, evidence suggests that eNOS localized to caveolae is spatially compartmentalized with the β-adrenoceptor and L-type $Ca^{2+}$ channel, allowing eNOS-generated NO to inhibit β-adrenoceptor-mediated contraction. Furthermore, the M2 muscarinic ACh receptor might also be coupled to eNOS, from which NO modulates ion channels that regulate pace making. Inhibition of eNOS prevents both the negative chronotropic effects of ACh receptor agonists and ACh-receptor-mediated inhibition of ICaL in pacemaking cells. Although the exact role played by eNOS in the cardiac myocyte is not firmly established, it is apparent that cellular microdomains of NOS isoforms are involved in a complex interplay in the regulation of cardiac ion channel function.

### NO and the Autonomic Control of the Heart Rate

As NO is well recognized as an endogenous neurotransmitter, neuromodulator, and intercellular messenger, it is expected that endogenous NO would be implicated in control of heart rate, acting at multiple sites including visceral afferents, brainstem neurons that mediate cardiovascular reflexes and cardiac autonomic ganglia. Because NO spreads very rapidly and freely from its source in nervous tissue, the question arises of how any sense can be made from this diffusible signal, which penetrates through any membrane and affects cells indiscriminately within a circumscribed area. To analyze this issue, the role of NO as a signaling molecule in the

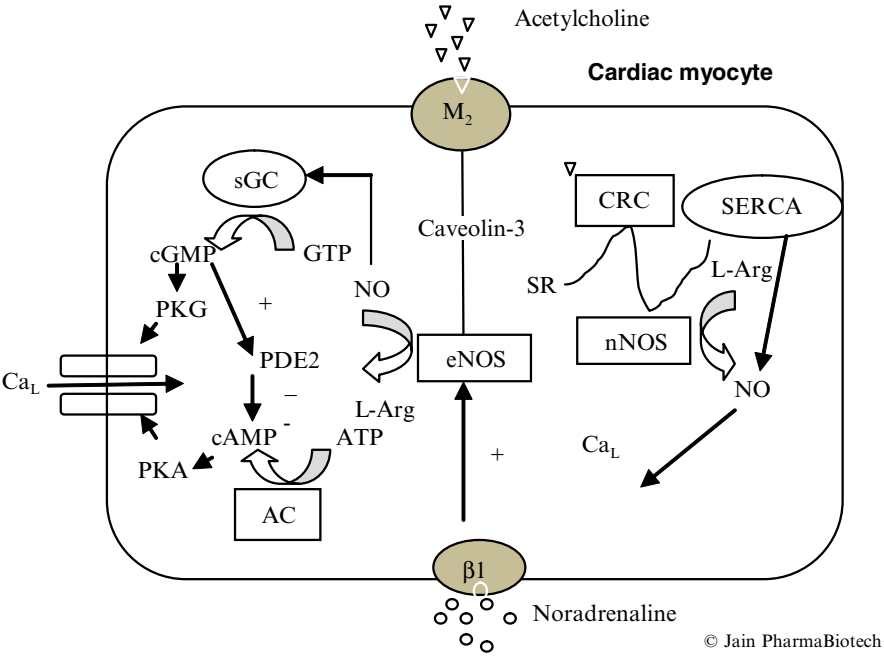


**Fig. 3.2** Role of NOS in functions of the cardiac myocyte. Postsynaptically, acetylcholine (Ach) binds to ACh receptors (M2) on the sinoatrial-node pacemaker cells and, via second messenger pathways, modulates ion channels to reduce heart rate. NO is generated in the pacemaker cell following M2-receptor activation via caveolin-3 and eNOS to inhibit flow of $Ca^{2+}$ through L-type Ca channels. When noradrenalin binds to the β1-adrenoceptor, nNOS localized in the sarcoplasmic reticulum can regulate $Ca^{2+}$ fluxes via SR-$Ca^{2+}$ ATPase (SERCA), $Ca_L$ and ryanodine-sensitive $Ca^{2+}$ release channels (CRC)] to minimize the effect of excessive sympathetic stimulation. *AC* adenylate cyclase, *PKA* protein kinase A, *PKG* protein kinase G, *PDE2* phosphodiesterase 2, *sGC* soluble guanylate cyclase

autonomic control of cardiac rate must be examined. Reflex control of cardiac rate depends on afferent input from various types of visceral afferent, with baroreceptors playing the most important role. It has been demonstrated that NO can affect peripheral afferent excitability but there are two other sites for NO modulation: the nucleus tractus solitarius (the brainstem termination site for baroreceptor afferents) and cardiac autonomic efferents at the level of the heart. At both these sites, there is extensive anatomical evidence to support sophisticated NO-mediated interactions.

## NO and Vasodilatation

NO relaxes the smooth muscle in the walls of the arterioles. At each systole, the endothelial cells that line the blood vessels release a puff of NO. This diffuses into the underlying smooth muscle cells causing them to relax and thus permit the surge

of blood to pass through easily. The NO/superoxide ($O_{2-}$) balance is also a key regulator of endothelial function. $O_{2-}$ levels are elevated in many forms of cardiovascular disease; therefore, decreasing $O_{2-}$ should improve endothelial function. Interactions between NO and the sympathetic nerve system, that is, norepinephrine, are of importance in the regulation of vascular tone. There is also a possible association of NO and endocannabinoid signaling with the vascular relaxation response, a physiological counterpart of the stress response. Because a fractional amount of guanylyl cyclase is sufficient to mediate vasorelaxation at higher NO concentrations, it is concluded that the majority of NO-sensitive guanylyl cyclase is not required for cGMP-forming activity but as NO receptor reserve to increase sensitivity toward the labile messenger NO in vivo (Mergia et al. 2006).

Mice whose genes for the eNOS found in endothelial cells has been "knocked out" suffer from hypertension. Nitroglycerine (glyceryl trinitrate, GTN), which is often prescribed to reduce the pain of angina, does so by generating NO, which relaxes the walls of the coronary arteries and arterioles. However, the identity of the cellular mechanisms through which GTN elicits NO-based signaling to dilate blood vessels remains controversial. Recent evidence suggests an unexpected role for mitochondria. Bioconversion by mitochondria of clinically relevant concentrations of GTN results in activation of guanylate cyclase, production of cGMP, vasodilation in vitro, and lowered blood pressure in vivo, which are eliminated by genetic deletion of the mitochondrial aldehyde dehydrogenase (mtALDH). Thus, mtALDH is required for vasoactivity derived from therapeutic levels of GTN and mitochondria can serve as a source of NO-based cellular signals that may originate independently of NOS activity (Chen et al. 2005). A genetically encoded high-sensitive indicator for visualizing physiological nanomolar dynamics of NO in living cells has shown that approx 1 nM of NO, which is enough to relax blood vessels, is generated in vascular endothelial cells even in the absence of shear stress (Sato et al. 2005). The nanomolar range of basal endothelial NO thus revealed appears to be fundamental to vascular homeostasis.

Several studies have attempted to determine whether NO is responsible for the "unexplained" limb vasodilation seen with body heating, limb ischemia, exercise, and mental stress. There are at least two independent mechanisms contributing to the rise in skin blood flow during nonpainful local heating: a fast-responding vasodilator system mediated by the axon reflexes and a more slowly responding vasodilator system that relies on local production of NO. Ultrasound induces vasorelaxation almost completely by time-dependent endothelial NO and prostacyclin release, which appears unrelated to tissue heating or endothelial architectural disruption. These observations are relevant to pain relief by application of heating pads to muscles and potential application for relief of intermittent claudication due to peripheral vascular insufficiency.

SIRT1 protein deacetylase mediates many of the effects of calorie restriction (CR) on organismal lifespan and metabolic pathways. Reduced caloric intake decreases arterial blood pressure in healthy individuals and improves endothelium-dependent vasodilation in obese and overweight individuals. A study has shown that SIRT1 promotes endothelium-dependent vasodilation by targeting eNOS for

deacetylation (Mattagajasingh et al. 2007). SIRT1 and eNOS colocalize and coprecipitate in endothelial cells, and SIRT1 deacetylates eNOS, stimulating eNOS activity and increasing endothelial NO. Inhibition of SIRT1 in the endothelium of arteries inhibits endothelium-dependent vasodilation and decreases bioavailable NO. Finally, CR of mice leads to deacetylation of eNOS. These results demonstrate that SIRT1 plays a fundamental role in regulating endothelial NO and endothelium-dependent vascular tone by deacetylating eNOS. Furthermore, this study provides a possible molecular mechanism connecting the effects of CR on the endothelium and vascular tone to SIRT1-mediated deacetylation of eNOS.

The molecular mechanisms of eNOS regulation of microvascular permeability remain unresolved. Agonist-induced internalization may have a role in this process. A study has demonstrated that internalization of eNOS is required to deliver NO to subcellular locations to increase endothelial monolayer permeability to macromolecules and suggests that a mechanism by which eNOS is activated by phosphorylation at the plasma membrane and its endocytosis is required to deliver NO to subcellular targets to cause hyperpermeability (Sánchez et al. 2009).

### Role of NO in the Plasma Compartment

The reaction products of NO with blood conserve its bioactivity and transduce an endocrine vasomotor function under certain conditions. Although S-nitrosated albumin has been considered the major species subserving this activity, additional NO species may also contribute. Human plasma consumes NO at a rate equivalent to that of hemoglobin (Hb). This NO consumption is mediated by the reaction of NO with plasma haptoglobin-hemoglobin complexes and limited slower reaction pathways required for S-nitrosation. Thus, high-affinity metal-based reactions in plasma with the haptoglobin-hemoglobin complex modulate plasmatic NO reaction products and limit S-nitrosation at low NO flux. Therefore, alternative NO reaction end products in plasma, such as nitrite, N-nitrosamines, iron-nitrosyls, and nitrated lipids, should be evaluated in blood NO transport along the vasculature.

It is proposed that the bond between NO and the Hb thiol Cys-β93 (SNOHb) is favored when Hb is in the relaxed (R, oxygenated) conformation, and that deoxygenation to tense (T) state destabilizes the SNOHb bond, allowing transfer of NO from Hb to form other (vasoactive) S-nitrosothiols (SNOs). $SNO_{RBC}$ is increased ≈ 20-fold in human septic shock and the $O_2$-dependent vasoactivity of RBCs is affected profoundly by SNO content in a murine lung bioassay indicating that SNO content and $O_2$ saturation are tightly coupled in intact RBCs (Doctor et al. 2005). This coupling is likely to be of pathophysiological significance.

### Measurement of NO as a Biomarker of Cardiovascular Function

The measurement of NO bioavailability is of great clinical interest in the assessment of cardiovascular health. However, NO is rapidly oxidized to form nitrite and nitrate and thus its direct detection in biological systems is difficult. Venous plasma nitrite (nM concentrations) has been shown to be a marker of forearm NO

production following pharmacological stimulation of the endothelium utilizing acetylcholine (Ach). In a study on healthy subjects, reactive hyperemia of the forearm, a physiological endothelial stimulus, results in a 52.5% increase in mean plasma nitrite concentrations (Allen et al. 2005). The subjects who could exercise hardest also produced the most NO. In contrast, subjects with cardiovascular disease showed no significant increases. However, plasma nitrite is readily oxidized to nitrate within plasma, and thus its utility as a marker of NO production within the clinical setting may be limited. Alternatively, NOx (predominantly nitrate) is relatively stable in plasma (microM concentrations), but is produced by sources other than the vasculature and has been shown to be unsuitable as a measure of localized NO production.

## *Hemoglobin, Oxygen, and Nitric Oxide*

Hb consists of four subunits that bind oxygen on heme-iron groups. It captures oxygen in the lungs and releases it in peripheral tissues. Hb also serves the function of discharging carbon dioxide, the cellular waste product of respiration. With the advent of NO research there is now abundant literature about the interaction of Hb with NO. The confinement of Hb within red blood cell (RBC) reduces the rate of reaction of Hb with NO by a factor of 1,000 because of two reasons:

1. The red cell membrane creates a barrier to the diffusion of NO.
2. A thin RBC-free zone, created by blood flow at the perimeter in vessels greater than 20 μm in diameter, separates NO from RBC and reduces the NO uptake rate.

It is generally accepted that Hb is crucial for oxidative inactivation of NO by reaction to nitrate and methemoglobin. The rapid destruction of NO by Hb raises the question of whether it is paracrine agent with only local effects or whether, like a hormone, it disseminates throughout the body. Various studies of reactions of NO with erythrocytes, plasma, and Hb, indicate that under normal physiological conditions, the reactions of NO with metals, heme groups, and free radicals predominate.

It is now recognized that NO senses oxygen levels and is an innate part of the body's oxygen delivery system. NO in blood cells is an active regulatory molecule that senses the oxygen level in tissue and causes Hb to undergo subtle shape changes to release its oxygen in tissues when levels are low but holds on to it when oxygen levels in tissue are high. Interactions of NO with Hb could regulate the uptake and delivery of oxygen by subserving the classical physiological responses of hypoxic vasodilation and hyperoxic vasoconstriction in the human respiratory cycle. In healthy adults alternately exposed to hypoxia or hyperoxia (to dilate or constrict arteries), binding of NO to hemes (FeNO) and thiols (SNO) of Hb varies as a function of $HbO_2$ saturation ($FeO_2$). RBC/SNO-mediated vasodilator activity is inversely proportional to $FeO_2$ over a wide range, whereas RBC-induced vasoconstriction correlates

directly with $FeO_2$. Thus, native RBCs respond to changes in oxygen tension ($pO_2$) with graded vasodilator and vasoconstrictor activity, which emulates the human physiological response subserving $O_2$ uptake and delivery. The ability to monitor and manipulate blood levels of NO, in conjunction with $O_2$ and carbon dioxide, may therefore prove useful in the diagnosis and treatment of many human conditions and in the development of new therapies. These results also help elucidate the link between RBC dyscrasias and cardiovascular morbidity.

Hb needs its natural partner in the blood, NO, to do its job of delivering oxygen to tissues, but current treatments deliver Hb without NO. Hb by itself actually reduces oxygenation to tissue because it constricts blood vessels, reducing blood flow. Thus, when delivered alone, hemoglobin may cause potentially fatal side effects and limit the effectiveness of radiation and chemotherapy. Some studies have shown that S-nitrosohemoglobin (SNO-Hb) behaves as a NO donor at low oxygen tensions. This property, in combination with oxygen transport capacity, suggests that SNO-Hb may have unique potential to reoxygenate hypoxic tissues. Simply addition of NO to the Hb can make it deliver more oxygen to tissues without boosting heart rate or constricting blood vessels (Sonveaux et al. 2005). These findings open up the possibility of using S-nitrosohemoglobin (SNO), combination of NO and Hb, therapeutically in patients where there is inadequate oxygen perfusion to tissue.

Nitrite anions comprise the largest vascular storage pool of NO, provided that physiological mechanisms exist to reduce nitrite to NO. In an experimental study, nitrite infusions were associated with rapid formation of erythrocyte iron-nitrosylated Hb and, to a lesser extent, S-nitroso-Hb (Cosby et al. 2003). NO-modified Hb formation was inversely proportional to oxyHB saturation. Vasodilation and formation of both NO gas and NO-modified Hb resulted from the nitrite reductase activity of deoxyHb and deoxygenated erythrocytes. This finding links tissue hypoxia, Hb, and nitrite bioactivation suggesting that nitrite represents a major bioavailable pool of NO. Thus, a new physiological function for Hb is that of a nitrite reductase, which potentially contributes to hypoxic vasodilation. These findings indicate that therapeutic application of nitrite should result in selective vasodilation of hypoxemic tissue and could be used to treat disease associated with ischemic tissue, neonatal pulmonary hypertension and hemolytic conditions such as sickle cell anemia where Hb released during hemolysis scavenges and disrupts NO-dependent vascular function. Nitrite would not only inhibit the ability of free Hb to scavenge NO by oxidizing it to methemoglobin but would also generate NO in tissue beds with low oxygen tension.

## Myoglobin and NO

Myoglobin (Mb) is a molecular relative of Hb and together these hemoproteins play vital roles in one of the most important aspects of animal metabolism: the acquisition and utilization of $O_2$. Mb not only is a key element determining the magnitude of the NO response in muscle but also plays an important role in overall NO inactivation in vivo. Experiments in myo(–/–) mice have shown that beyond its function in $O_2$ supply Mb substantially contributes to NO homeostasis in the heart

(Flögel et al. 2010). They found that decrease in tissue $O_2$ tension drives the conversion of Mb from being a NO scavenger under normoxia to a NO producer during hypoxia, and mitochondrial respiration is reversibly adapted to the intracellular $O_2$ tension. Therefore, Mb may act as an important $O_2$ sensor through which NO can regulate muscle energetics and function. It was concluded that Mb's multifunctional properties may create an environment characterized by a tightly adapted aerobic mitochondrial respiration and low levels of free radicals, and thus serve an essential and beneficial role within the myocardium.

## *NO and Pulmonary Circulation*

In contrast to systemic vascular smooth muscle, pulmonary vascular smooth muscle constricts in response to local hypoxia. This is because of the basic differences between the two circulatory systems. The goal of systemic circulation is to deliver oxygen to those tissues most in need, whereas pulmonary circulation aims to match ventilation and perfusion for optimal alveolar gas exchange. Thus local hypoxia anywhere in the systemic circulation results in vasodilation and increased blood flow to the hypoxic regions. On the other hand, hypoxic pulmonary vasoconstriction acts to divert blood away from hypoxic lung regions. This minimizes the effect of lung pathology on the arterial $PO_2$ by reducing blood flow to the poorly ventilated, hypoxic alveoli. The pulmonary vasculature responds predominantly to alveolar rather than circulatory hypoxia and the response is rapid with rise of pulmonary artery pressure. There are several proposed mechanisms for the initiation of hypoxic pulmonary vasoconstriction. According to one of these hypotheses, hypoxia increases free radical production and activates K channels in systemic arteries, but it decreases free radical production and inhibits K channels in the pulmonary circulation. Various oxygen sensor mechanisms to explain this difference are not satisfactory. This has focused attention on the role of NO and hemoglobin on hypoxic pulmonary vasoconstriction.

### Role of NO in the Regulation of Hypoxic Pulmonary Vasoconstriction

Basal release of NO from pulmonary vascular endothelium and airway epithelium acts to moderate hypoxic pulmonary vasoconstriction through activation of guanylate cyclase and production of cGMP. Hypoxic pulmonary vasoconstriction is markedly enhanced by NOS and is inhibited by inhalation of NO. NOS enzyme activity is dependent on oxygen concentration. Therefore, a fall in NO production during hypoxia due to reduced NOS activity may enhance pulmonary vasoconstriction. Another explanation for the pulmonary vasoconstriction despite continuous NO production lies within the vascular lumen in the form of red blood cells (RBCs) because hemoglobin contained in these cells inactivates NO. This interaction between RBCs, NO, and the pulmonary circulation is critical in understanding the effects of anemia and polycythemia on pulmonary blood flow distribution, gas exchange, and global oxygen delivery and in understanding the development of hemoglobin-based oxygen carriers. Pharmacologic (cross-linking) modification of

hemoglobin involved in the construction of hemoglobin-based oxygen carriers is limited by their increased capacity to produce pulmonary vasoconstriction by NO scavenging action.

## Role of NO in Pathomechanism of Cardiovascular Disorders

### *Oxidative Stress as a Cause of Cardiovascular Disease*

Excessive production and/or inadequate removal of ROS, especially superoxide anion, have been implicated in the pathogenesis of many cardiovascular diseases, including atherosclerosis, hypertension, diabetes, and in endothelial dysfunction by decreasing NO bioactivity. Nanosensing techniques have been developed to monitor the physiology of NO in the beating heart in vivo. These methods involve the application of nanosensors to monitor real-time dynamics of NO production in the heart as well as the dynamics of oxidative species (oxidative stress) produced in the failing heart (Malinski 2005). Results of studies using nanotechnology demonstrated that African Americans have an inherent imbalance of NO, $O_{2^-}$, and ONOO- production in the endothelium (Jain 2011a). The overproduction of $O_{2^-}$ and ONOO- triggers the release of aggressive radicals and damages cardiac muscle (necrosis), which may explain why African Americans are at greater risk for developing cardiovascular diseases, such as hypertension and heart failure, and are more likely to have complications than European Americans. Potential therapeutic strategies to prevent or ameliorate damage to the heart during cardiac events are prevention of $O_{2^-}$ and ONOO- production, supplementation of NO (NO donors), and scavenging of $O_{2^-}$ (antioxidants)

Since the vascular levels of $O_{2^-}$ are regulated by the superoxide dismutase (SOD) enzymes, a role of SOD in the cardiovascular disease is of substantial interest. A major form of SOD in the vessel wall is the extracellular SOD.

Vascular aging may be related to oxidative stress. Age-related morphologic changes in large resistance vessels include an intima-media thickening, increased deposition of matrix substances, thus ultimately leading to a reduced compliance. Vascular aging is mainly characterized by an impaired endothelium-dependent vasorelaxation. The expression of endothelial nitric oxide synthase (eNOS), producing vasodilatatory NO, is markedly upregulated with increasing age. However, vasorelaxation is impaired as the production of ROS, such as superoxide ($O_{2^-}$), concomitantly increases.

### *Role of NO in Pathomechanism of Cardiovascular Diseases*

Interactions between superoxide and NO underlie many physiologic and pathophysiologic processes. Xanthine oxidoreductase (XOR), a prototypic superoxide producing enzyme, and nNOS coimmunoprecipitate and colocalize in the sarcoplasmic reticulum of cardiac myocytes. Deficiency of nNOS (but not eNOS) leads to pro-

found increases in XOR-mediated superoxide production, which in turn depresses myocardial excitation-contraction coupling in a manner reversible by XOR inhibition with allopurinol (Khan et al. 2004). These data demonstrate a unique interaction between a NO and a superoxide generating enzyme that accounts for crosstalk between these signaling pathways; these findings demonstrate a direct antioxidant mechanism for nNOS and have pathophysiologic implications for the growing number of disease states in which increased XOR activity plays a role.

There is compelling evidence for vasoprotective actions of NO, which are mediated by cGMP-dependent, and cGMP-independent mechanisms. Various vasoprotective mechanisms are:

- Vasodilator effect
- Antiplatelet effect
- Antiadhesive effect
- Antioxidant effect

These effects may contribute to the beneficial effects of established drugs such as ACE inhibitors or statins. Pharmacological compounds that release NO have been useful tools for evaluating the pivotal role of NO in cardiovascular physiology and therapeutics. These agents constitute two broad classes of compounds, those that release NO or one of its redox congeners spontaneously and those that require enzymatic metabolism to generate NO. In addition, several commonly used cardiovascular drugs exert their beneficial action, in part, by modulating the NO pathway. Unfortunately, clinical data on the effect of long-term treatment with nitrates on the progression of coronary artery disease are lacking. Finally, L-arginine or new activators of the NO pathway may become therapeutic options in the future.

### Role of iNOS in Cardiovascular Disease

Expression of the inducible form of nitric oxide synthase (iNOS) has been reported in a variety of cardiovascular diseases. The resulting high output NO formation, besides the level of iNOS expression, depends also on the expression of the metabolic pathways providing the enzyme with substrate and cofactor. Besides selectively inhibiting iNOS, a number of other therapeutic strategies are conceivable to alleviate deleterious effects of excessive NO formation, including peroxynitrite (ONOO-) scavenging and inhibition of metabolic pathways triggered by ONOO-. When available, these approaches might have the advantage to preserve beneficial effects of iNOS induction. Counteracting vascular hyper-responsiveness to endogenous vasoconstrictor agonists in septic shock, or inducing cardiac protection against ischemia-reperfusion injury are examples of such beneficial effects of iNOS induction.

### Role of eNOS in Cardiovascular Disease

Endogenous eNOS-derived NO exerts direct effects in preserving blood flow, thereby promoting arteriogenesis, angiogenesis, and mural cell recruitment to

immature angiogenic sprouts (Yu et al. 2005). The genetic loss of eNOS in mice impairs vascular endothelial growth factor (VEGF) and ischemia-initiated blood flow recovery resulting in critical limb ischemia. An experimental study has shown that the impaired neovascularization in mice lacking eNOS is related to a defect in VEGF-induced progenitor cell mobilization (Aicher et al. 2003). Intravenous infusion of wild-type progenitor cells, but not bone marrow transplantation, rescued the defective neovascularization. These findings indicate that eNOS expressed by bone marrow stromal cells influences recruitment of stem and progenitor cells. This may contribute to impaired regeneration processes in ischemic heart disease patients, who are characterized by a reduced systemic NO bioactivity.

During the last two decades it has become apparent that a variety of diseases are associated with an impairment of endothelium-dependent NO activity. One of the major causes is believed to be an increased production of reactive oxygen species, in particular superoxide, which has been shown to interfere with many steps of the NO-cyclic guanosine monophosphate (cGMP) pathway. This phenomenon has been found in diverse conditions such as atherosclerosis, hypertension, diabetes, hypercholesterolemia, heart failure, and cigarette smoking.

African Americans suffer from cardiovascular diseases at a rate about five times higher than the rest of the US population. The culprit is a serious deficiency of NO (Kalinowski et al. 2004). ONOO- plays a central role in vascular pathophysiology. The differences in endothelial NO/$O_2$-/ONOO- metabolism may highlight the potential predisposition to endothelial dysfunction and cardiovascular complications prevalent in blacks. NO/$O_2$-/ONOO- nanosensors were used to record eNOS from endothelial cell isolated from blacks and whites. Compared to whites, endothelial cells from blacks elicited reduced release of bioactive NO with an accompanying increase in the release of both $O_2$- and ONOO-. The greater potency of NO production because of eNOS upregulation in blacks is associated with a decrease in the NO bioavailability. This is due to increased NO degradation by excess $O_2$- produced primarily by two enzymatic sources: NAD(P)H-oxidase and uncoupled eNOS. Compared with whites, the steady-state NO/$O^2$-/ONOO- balance in endothelial cells from blacks is kept closer to the redox states characteristic for the endothelium-impaired function disorders. This may explain the differences in racial predisposition to the endothelium dysfunction during ongoing vascular disturbances with the hallmark of enhanced NO inactivation within the endothelium by oxidative stress. This observation opens the door for the development of new drugs designed specifically to maintain healthy levels of NO in the cardiovascular system.

### Role of nNOS in Cardiac Arrhythmia and Sudden Death

NO derived from nNOS facilitates cardiac vagal neurotransmission and bradycardia in vitro. Pre-/post-ganglionic synapse is considered to be a site for NO-mediated facilitation of vagal bradycardia. Functional gene expression induced with adenoviral vectors may provide a novel intervention to acutely modulate the neural control of cardiac excitability.

Extremes of the electrocardiographic QT interval, a measure of cardiac repolarization, are associated with increased cardiovascular mortality. A gene called

NOS1AP that may predispose some people to abnormal heart rhythms leading to sudden cardiac death was identified through a genome-wide association study (Arking et al. 2006). NOS1AP, a regulator of nNOS, modulates cardiac repolarization. The gene appears to influence significantly QT interval length as risk factor for sudden cardiac death.

## *NO and Atherosclerosis*

Atherosclerosis is a noninflammatory degenerative disease that can affect segments of almost any artery in the body. It is particularly important in the pathogenesis of diseases of the heart and the cerebrovascular system. Disturbances of blood flow in arteries also play a role in pathogenesis of atherosclerosis. In addition to triggering the immediate release of NO, laminar shear stress increases the expression of NOS and that of SOD (which reduces oxidative degradation of NO). Thus, in a physiological range, laminar shear stress maintains vascular homeostasis. By contrast, disturbed flow (causing low and oscillating shear stress) inhibits the release of these factors. At the branches of coronary arteries in humans, endothelium-dependent vasodilatation is impaired. Similarly, when cultured endothelial cells are exposed to disturbed flow, NOS expression is not upregulated as when endothelial cells are exposed to laminar flow. This hemodynamic alteration of endothelial biology predisposes to processes involved in atherogenesis. The balance between vascular generation of two free radicals, NO and $O_2$, is a major determinant of endothelial adhesiveness and lesion formation.

Many studies have shown that NO is a major antioxidant that serves to block oxygen-free radicals, which, among other things, create oxidized low-density lipoprotein (LDL) cholesterol that damages the endothelium and leads to atherosclerosis. The protective role of red wine in endothelial function might be due, in large part, to the three-fold increase NO observed with the flavanol intake.

Generation of the second-messenger molecule ceramide by stimulated sphingomyelinase activity has been implicated in the inflammatory processes contributing to the pathogenesis of atherosclerosis. Ceramide produces oxidative stress in human endothelial cells, thereby reducing bioactive NO. The partial reversal of this reduction by $BH_4$ and the diminution of ROS generation by L-NAME suggest that ceramide promotes NADPH oxidase activity of eNOS, leading to ROS formation at the expense of NO synthesis. The ceramide-induced upregulation of eNOS gene transcription can be considered an ineffective compensatory mechanism. The decreased bioavailability of NO is likely to favor a proatherogenic role of ceramide.

Endothelial dysfunction due to impaired L-arginine/NO pathway is an early feature of atherosclerosis, which often leads to changes in endothelial redox state, activation of oxidant-sensitive transcriptional genes (e.g., monocyte chemoattractant protein-1, vascular cell adhesion molecule), adhesion of circulating platelets and monocytes, increased accumulation of lipids in the intima, and increased contraction, migration, and proliferation of smooth muscle cells. Progressive develop-

ment of atherosclerotic lesions results in the formation of lipid-laden plaques that are prone to fissure, ulceration, and rupture. Thrombosis resulting from the plaque rupture in the coronary arteries plays a pivotal role in the progression from atherosclerosis to myocardial infarction. Hence, NOS therapy might be expected to improve endothelium-dependent vasodilation, reverse the altered endothelial redox state, suppress smooth muscle cell proliferation, and stabilize vulnerable plaques. Ingestion of certain NO-boosting substances, including L-arginine, L-citrulline, and antioxidants has been shown to reduce oxidative stress and reverse the progression of atherosclerosis in rabbits fed a high-cholesterol diet (Hayashi et al. 2005). This approach may have clinical utility in the treatment of atherosclerosis in humans.

The impaired endothelium-dependent vasodilatory response (EDR) has been demonstrated in vessels exposed to hypercholesterolemia and atherosclerosis. The extent of impairment serves as a predictor of future progression of atherosclerosis. As to the mechanisms of impaired EDR, increased production of superoxide is important and is linked to eNOS. eNOS becomes dysfunctional and produces superoxide rather than NO under conditions in which vascular tissue levels of tetrahydrobiopterin ($BH_4$), a cofactor for eNOS, are deficient or lacking. Dysfunctional eNOS is closely implicated in the endothelial dysfunction represented by impaired EDR in various vascular disorders including atherosclerosis. Experimental studies in vitro reveal that NO from eNOS constitutes as an anti-atherogenic molecule. In eNOS-knockout mice, eNOS deficiency augments atherosclerotic lesion formation, although the effects may be partly due to the associated hypertension. However, in eNOS-transgenic mice (eNOS-Tg) crossbred with apolipoprotein E–deficient mice (apoE-KO/eNOS-Tg), the accelerated lesion formation in association with increased superoxide production from vessels was comparable to that in apoE-KO mice (Kawashima 2004). Chronic administration of exogenous $BH_4$ or overexpression of GTPCH-1, a rate limiting enzyme for $BH_4$ synthesis, restored the lesion to the levels comparable to apoE-KO mice. Therefore, eNOS may have two faces in the pathophysiology of atherosclerosis depending on tissue $BH_4$ levels.

## *Role of NO in Cardiopulmonary Disorders*

Red blood cells (RBCs) act as $O_2$-responsive transducers of vasodilator and vasoconstrictor activity in lungs and tissues by regulating the availability of NO. Vasodilation by RBCs is impaired in diseases characterized by hypoxemia. The extent to which RBCs constrict versus dilate vessels is, at least partly, controlled by a partitioning between NO bound to heme iron and to Cysβ93 thiol of Hb. Hemes sequester NO, whereas thiols deploy NO bioactivity. Specific micropopulations of NO-liganded Hb could support the chemistry of S-nitrosohemoglobin (SNO-Hb) formation. By using nitrite as the source of NO, it was demonstrated that a micropopulation of a heme-NO species, with spectral and chemical properties of $Fe_3NO$, acts as a precursor to SNO-Hb formation, accompanying the allosteric transition of Hb to the R state (Angelo et al. 2006). At physiological concentrations of nitrite

and deoxyHb, a S-nitrosothiol precursor is formed within seconds and produces SNO-Hb in high yield upon its prompt exposure to $O_2$ or CO. Deoxygenation/reoxygenation cycling of oxyHb in the presence of physiological amounts of nitrite also efficiently produces SNO-Hb. In contrast, high amounts of nitrite or delays in reoxygenation inhibit the production of SNO-Hb. These data provide evidence for a physiological S-nitrosothiol synthase activity of tetrameric Hb that depends on NO-Hb micropopulations and suggest that dysfunction of this activity may contribute to the pathophysiology of cardiopulmonary and blood disorders.

## *Role of NO in Disturbances of Vasodilation*

NO physiologically stimulates the sarco/endoplasmic reticulum calcium ($Ca^{2+}$) ATPase (SERCA) to decrease intracellular $Ca^{2+}$ concentration and relax cardiac, skeletal, and vascular smooth muscle. NO-derived peroxynitrite (ONOO-) directly increases SERCA activity by S-glutathiolation and this modification of SERCA is blocked by irreversible oxidation of the relevant cysteine thiols during atherosclerosis. Because superoxide scavengers decrease S-glutathiolation of SERCA and arterial relaxation by NO, ONOO- is implicated as the intracellular mediator. NO-dependent relaxation as well as S-glutathiolation and activation of SERCA is decreased by atherosclerosis. Thus, irreversible oxidation of key thiol(s) in disease impairs NO-induced relaxation by preventing reversible S-glutathiolation and activation of SERCA by NO/ONOO-. These results suggest a new concept regarding the role of superoxide in NO bioavailability. A certain amount of intracellular superoxide may be required for NO signaling. Pathologically enhanced extracellular superoxide scavenges NO and decreases its diffusion into the smooth muscle. Therefore, one benefit of chronic antioxidant therapy may result from maintaining levels adequate for cell signaling while preventing irreversible protein thiol oxidation.

## *Role of NO in Hypercholesterolemia*

Relatively brief periods (days) of hypercholesterolemia can exert profound effects on endothelium-dependent functions of the microcirculation, including dilatation of arterioles, fluid filtration across capillaries, and regulation of leukocyte recruitment in postcapillary venules. Hypercholesterolemia appears to convert the normal anti-inflammatory phenotype of the microcirculation to a proinflammatory phenotype. This phenotypic change appears to result from a decline in NO bioavailability that results from a reduction in NO biosynthesis, inactivation of NO by superoxide ($O_{2-}$), or both. A consequence of the hypercholesterolemia-induced microvascular responses is an enhanced vulnerability of the microcirculation to the deleterious effects of ischemia and other inflammatory conditions. During inflammation, leukocytes interact with the vascular endothelium in three steps: an initial slow, rolling phase, then firm adhesion, followed by transendothelial migration as shown in Fig. 3.3.

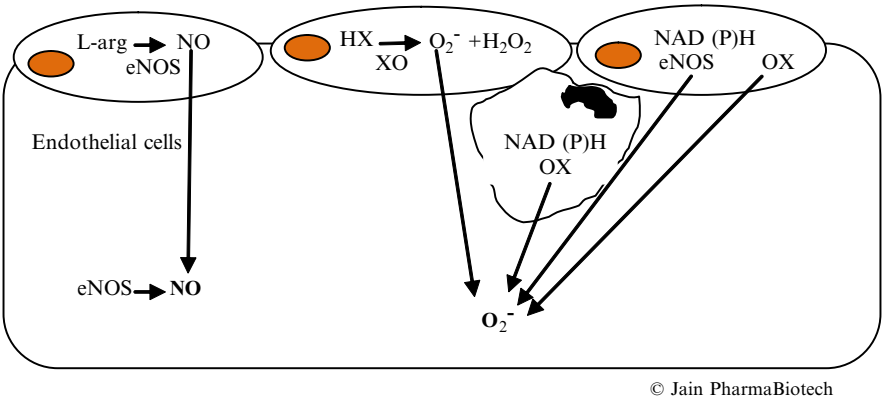


**Fig. 3.3** Blood cell–endothelial cell interactions induced by hypercholesterolemia. The interactions are modulated by both NO (produced by eNOS) and $O_2$, which is produced by multiple sources including hypoxanthine (*HX*) via xanthine oxidase (*XO*) in endothelial cells and NAD(P) Hox in both leukocytes and endothelial cells

In an experimental study, mice who were fed high cholesterol diet, received either nitrite-free drinking water or supplemental nitrite in their drinking water (Stokes et al. 2009). The results showed that mice fed a cholesterol-enriched diet exhibited significantly elevated leukocyte adhesion to and emigration through the venular endothelium as well as impaired endothelium-dependent relaxation in arterioles. Administration of nitrite in the drinking water inhibited this process and prevented the arteriolar dysfunction. These findings support novel anti-inflammatory properties of nitrite and provide a rationale for the use of nitrite as a therapy for microvascular inflammation and endothelial dysfunction associated with hypercholesterolemia.

Hence, therapeutic strategies that are directed toward preventing the early microcirculatory dysfunction and inflammation caused by hypercholesterolemia may prove effective in reducing the high mortality associated with ischemic tissue diseases. Agents that act to maintain the normal balance between NO and reactive oxygen species (ROS) in vascular endothelial cells may prove particularly useful in this regard.

Another study has shown that severe hypercholesterolemia leads to atheromatous lesion formation following vascular injury (Raman et al. 2011). However, iNOS gene transfer still effectively inhibits atheroma formation. These findings support early correction of hypercholesterolemia and emphasize the potential role for NO-based therapies in cardiovascular diseases.

## *Pulmonary Hypertension*

Pulmonary hypertension is a progressively deteriorating syndrome characterized by an increase in pulmonary vascular tone and reactivity and proliferation of pulmonary vascular smooth muscle cells with thickening of arterial vessel walls, resulting in a

marked increase in vascular resistance and onset of right ventricular hypertrophy leading to right-sided heart failure and death. Central to the pathogenesis of pulmonary hypertension may be endothelial cell dysfunction, leading to imbalances in vasodilator (decreased prostacyclin and NO) versus vasoconstrictors (increased endothelin). Current approaches for treating pulmonary hypertension include supplemental oxygen, anticoagulants and vasodilator drugs (calcium channel blockers, prostacyclin), and inhaled NO.

Immunohistochemistry studies have localized NOS in pulmonary nerves, airway epithelium and in pulmonary vascular cells, suggesting a role for NO in pulmonary homeostasis. Endothelial NO-dependent relaxation of pulmonary arteries has been reported to be impaired in other forms of pulmonary hypertension. The impaired NO-dependent relaxation may result from several causes, including:

- Direct damage and/or loss of endothelium
- Reduced NO production secondary to impaired arginine uptake or decreased NO synthase activity
- Accelerated inactivation NOS mRNA, NOS, or NO
- Reduced cGMP production secondary to impaired guanylate cyclase activity

In an ovine model of persistent pulmonary hypertension of the newborn, endothelin-1 (ET-1) expression is increased, while eNOS expression is decreased. In a study on pulmonary arterial endothelial cells isolated from fetal lambs, ET-1 secretion is increased by overexpression of Prepro-ET-1, a precursor of ET-1 (Sud and Black 2009). This results in activation of PKCδ, which phosphorylates STAT3, increasing its binding to the eNOS promoter. This in turn decreases eNOS promoter activity, protein levels, and NO production. Thus, ET-1 can reduce eNOS expression and NO generation in fetal pulmonary artery endothelial cells through PKCδ-mediated activation of STAT3.

## *NO and Systemic Hypertension*

Hypertension, a major cardiovascular risk factor and cause of mortality worldwide, is thought to arise from primary renal abnormalities. However, the cause of most cases of hypertension is unknown. Vascular tone, an important determinant of blood pressure, is regulated by NO, which causes vascular relaxation by increasing intracellular cGMP and activating cGMP-dependent protein kinase I (PKGI). A study has shown that mice with a selective mutation in the N-terminal protein interaction domain of PKGIα display inherited vascular smooth muscle cell abnormalities of contraction, abnormal relaxation of large and resistance blood vessels, and increased systemic blood pressure (Michael et al. 2008). Renal function studies and responses to changes in dietary sodium in the PKGIα mutant mice are normal. These data reveal that PKGIα is required for normal VSMC physiology and support the idea that high blood pressure can arise from a primary abnormality of

vascular smooth muscle cell contractile regulation, suggesting a new approach to the diagnosis and therapy of hypertension and cardiovascular diseases.

A functional imbalance between angiotensin II (Ang II) and NO plays an important pathogenetic role in hypertensive end-organ injury. NO, an endogenous vasodilator, inhibitor of vascular smooth muscle and mesangial cell growth, and natriuretic agent, is synthesized in the endothelium by a cNOS. NO antagonizes the effects of Ang II on vascular tone, cell growth, and renal sodium excretion, and also downregulates the synthesis of angiotensin-converting enzyme (ACE) and Ang II type 1 receptors. On the other hand, Ang II decreases NO bioavailability by promoting oxidative stress. A better understanding of the pathophysiologic mechanisms involved in hypertensive end-organ damage may aid in identifying biomarkers of cardiovascular susceptibility to injury and in developing therapeutic interventions. Antihypertensive agents that lower blood pressure and concomitantly restore the homeostatic balance of vasoactive agents such as Ang II and NO within the vessel wall would be more effective in preventing or arresting end-organ disease.

Among various mediators and conditions, stress is the most important factor in the release of NO in the general circulation. Another signal for the release of NO in the circulation is bradykinin. NO has a greater role in the maintenance of vascular resistance in the kidney than in other tissues. Glomerular filteration, one of the basic functions of the kidney, is critically determined by the balance of vascular tone between the afferent and efferent arterioles. NO blockade causes systemic hypertension.

The use of enalapril is not completely efficient in reducing BP and morphological injury when the hypertension of spontaneous hypertensive rats is increased with the NOS blockade suggesting that hypertension induced by NO deficiency is not entirely mediated by the renin-angiotensin-aldosterone system (Barbuto et al. 2004).

An open, comparative, crossover 8-week trial of beta-blockers, nebivolol and metoprolol, in patients with arterial hypertension studied the ambulatory blood pressure and heart rate levels, endothelium-dependent vasorelaxation, endothelial NOS, and NO production (Buval'tsev et al. 2003). It was found that nebivolol activated the system eNOS-NO and improved vasomotor parameters of the endothelium. Metoprolol failed to activate enzyme eNOS activity and NO production to the same level and did not improve the endothelial vasomotor function. The antihypertensive activity of nebivolol was higher than that of metoprolol. Thus, nebivolol can be used in patients with hypertension and cardiovascular risk factors to correct endothelial dysfunction.

In clinical studies of hypertensive subjects, nebivolol significantly improves vasodilator responses to endothelium-dependent agonists such as acetylcholine. In addition, nebivolol significantly reduces pulse wave velocity (PWV), a measure of arterial stiffness, whereas the beta-blocker atenolol has no effect on PWV. Because endothelial dysfunction and arterial stiffness play an integral part in the early atherosclerotic process and are associated with poor outcomes and increased mortality, independent of blood pressure, the ability of nebivolol to enhance release of endothelium-derived NO may have significant clinical implications for the use of this agent in the treatment of hypertension.

## *Coronary Artery Disease*

There is compelling evidence that the endothelium is critical to normal coronary vascular function and that endothelial dysfunction, generally indicated by an impairment of endothelium-dependent vasodilatation, is an important component of coronary artery disease (CAD). Endothelial cells synthesize and release a number of factors, including prostacyclin, NO, endothelium-derived hyperpolarizing factor, and endothelin, which are important in the regulation of vascular tone and the control of platelet and leukocyte adhesion, aggregation, and migration. NO appears to be the critical factor in the preservation of normal coronary vascular function and there is an established correlation between CAD and an impairment of NO activity. Thus, to preserve endothelial function, drugs have been used to either increase the synthesis of NO, or to decrease its breakdown.

The eNOS gene harbors a common polymorphism in intron 4 (4a/b), and some clinical studies have suggested an association of the rare a-allele with coronary artery disease and myocardial infarction. However, contradictory results have also been reported, for example, no significant differences in areas of atherosclerotic lesions and coronary stenosis percentages were found between men carrying the a-allele (ba + aa) compared with those homozygous for the b-allele. Subjects with the a-allele had significantly lower risk of myocardial infarction compared with those carrying the bb genotype. Men with the a-allele also tended to have coronary thrombosis less often. The eNOS gene 4a/b polymorphism is not associated with the extent of coronary atherosclerosis, but the a-allele of the variant seems to protect to some degree against the development of myocardial infarction.

## *Role of NO in the Pathophysiology of Angina Pectoris*

Coronary artery spasm plays an important role in the pathogenesis of vasospastic angina, and contributes to the development of several acute coronary syndromes. eNOS catalyzes the synthesis of NO, which regulates vascular tone, and may be related to coronary vasospasm. Coronary spasm may be related to particular polymorphisms of the eNOS gene. Both a/a and a/b genotypes in intron 4 of eNOS (NOS4a) are significant predictors of coronary spasm (Kaneda et al. 2006). In patients with NOS4a, both the induced and spontaneous contractions are augmented indicating that NOS4a could be a good biomarker for coronary artery spasm.

Polymorphism of eNOS T-786C gene is associated with reduced NO production and coronary artery spasm and it might be associated with Prinzmetal's variant angina. In a study, PCR analyses of eNOS T-786C and stromelysin 5A6A polymorphisms were done in white as well as black American women and men with well-documented Prinzmetal's variant angina and each case was matched with a healthy control by race and gender (Glueck et al. 2010). Patients did not differ from controls for the distribution of the stromelysin 6A mutation or for the mutant 6A allele frequency. Effect of NO-elevating L-arginine was tested on relief of angina in these

patients. The study concluded that eNOS T-786C mutation appears to be a reversible etiology of Prinzmetal's variant angina in white Americans whose angina might be ameliorated by L-arginine.

## *Role of NO in the Pathophysiology of Congestive Heart Failure*

The clinical syndrome of CHF is characterized by abnormalities of left ventricular function and neurohormonal regulation, which are accompanied by effort intolerance, fluid retention, and decreased longevity. While an increased sympathetic tone and an activated renin-angiotensin system may contribute to the reduced vasodilatory capacity in patients with CHF, the important role of the endothelium in coordinating tissue perfusion has now been recognized. CHF is associated with endothelial dysfunction, as demonstrated by impaired endothelium-mediated vasodilation. Endothelial dysfunction in patients with CHF is a critical component in the systemic vasoconstriction and reduced peripheral perfusion that characterizes these patients. Endothelial regulation of vascular tone is mediated mainly by NO. Increased oxidative stress in patients with CHF is likely caused by decreased bioavailability of NO due to reduced expression of eNOS and increased generation of ROS. These react with NO in the setting of decreased antioxidant defenses that would normally clear these radicals, culminating in attenuated endothelium-dependent vasodilation in patients with CHF. Therapies that improve endothelial function have been shown to improve exercise tolerance and outcomes in patients with CHF. Endothelial dysfunction is thus an important target for future therapy in patients with CHF.

### Calcium Overload as a Cause of Heart Failure

Two NO-producing enzymes are involved in modulation of excitation-contraction in cardiac myocytes: (1) eNOS, which is preferentially localized to the caveolae of the cell membrane where it is associated with the structural protein caveolin-3 (Cav-3); and (2) nNOS, which is preferentially localized in the sarcoplasmic reticulum, where it is associated with the $Ca^{2+}$ channel ryanodine receptor (RyR2) and the enzyme xanthine oxyreductase (XOR). NO produced by nNOS regulates the function of RyR2 by S-nitrosylation, and suppresses the activity of XOR, thereby reducing the production by this enzyme of ROS. Contraction of cardiac myocytes is initiated by the influx of $Ca^{2+}$ through the L-Type $Ca^{2+}$ channel during the plateau of the action potential, which triggers the release of $Ca^{2+}$ from the SR through the RYR2. The elevated level of $Ca^{2+}$ in the cytoplasm facilitates the contraction of the myofilaments. $Ca^{2+}$ is pumped back into the SR by the SR $Ca^{2+}$ ATPase (SERCA). In heart failure, nNOS partially translocates to the cell membrane resulting in loss of RyR2 regulation and increased production of $O_{2-}$, which makes RYR2 leaky. Sustained elevated cytoplasmic level of $Ca^{2+}$ (calcium overload) decreases the myofilament $Ca^{2+}$-sensitivity resulting in poor myocardial contractility, a characteristic of the failing heart.

### NO/Redox Disequilibrium in the Failing Heart

CHF is a consequence of NO/redox disequilibrium in the cardiovascular system. The altered production and/or spatiotemporal distribution of ROS and nitrogen species creates oxidative and/or nitrosative stresses in the failing heart and vascular tree, which contribute to the abnormal cardiac and vascular phenotypes that characterize the failing cardiovascular system. These derangements at the integrated system level can be interpreted at the cellular and molecular levels in terms of adverse effects on signaling elements in the heart, vasculature, and blood that subserve cardiac and vascular homeostasis. CDP-1050 (Cordex Pharma) corrects NO and redox imbalance in the failing heart and the cardiovascular system and is in a phase II clinical trial.

## *Myocardial Ischemia/Reperfusion Injury*

Myocardial ischemia, resulting mainly from atherosclerotic coronary artery disease, is the most common pathology in cardiology and is a major cause of morbidity and mortality in the developed world. A major component of ischemia is the reduction of oxygen supply (hypoxia), but other disturbances such as reduced delivery of substrates and accumulation of tissue metabolites also play a part.

Reperfusion after the onset of ischemia apparently restores the blood supply but it can have deleterious effects including the following:

- Coronary endothelial dysfunction
- Adherence of neutrophils to the endothelium
- Impaired myocardial blood flow ("no-reflow" phenomenon)
- Ischemic cell swelling leading to necrosis, hemorrhage, and extension of infarct
- Postischemic depression of contractility
- Arrhythmias

The mechanism of reperfusion injury includes the following:

- Generation of ROS, particularly $O_{2-}$ following reperfusion, which interact with NO. The removal of $O_{2-}$ by reaction with NO and formation of $ONOO^{-}$ may be considered beneficial. However, the protonation of $ONOO^{-}$ may form peroxynitrous acid, a strong oxidant.
- Induction of cellular calcium overload.
- Hypercontraction of myocytes following reenergization.

The nitrite anion is reduced to NO as oxygen tension decreases. Whereas this pathway modulates hypoxic NO signaling and mitochondrial respiration and limits myocardial infarction in mammalian species, the pathways to nitrite bioactivation remain uncertain. Studies suggest that hemoglobin and myoglobin may subserve a fundamental physiological function as hypoxia-dependent nitrite reductases.

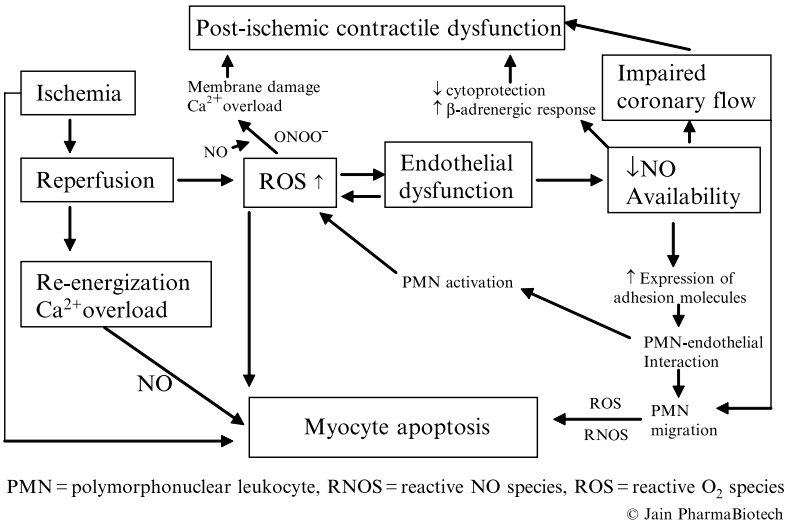


**Fig. 3.4** Effects of NO on the pathophysiology of myocardial ischemia-reperfusion. *PMN* polymorphonuclear leukocyte, *RNOS* reactive NO species, *ROS* reactive $O_2$ species

Myoglobin is responsible for nitrite-dependent NO generation and cardiomyocyte protein iron-nitrosylation. Nitrite reduction to NO by myoglobin dynamically inhibits cellular respiration and limits ROS generation and mitochondrial enzyme oxidative inactivation after ischemia/reperfusion injury. In vivo administration of nitrite reduces myocardial infarction by 61% in myoglobin+/+ mice, whereas in myoglobin−/− mice nitrite has no protective effects (Hendgen-Cotta et al. 2008). These data support an emerging paradigm that myoglobin and the heme globin family subserve a critical function as an intrinsic nitrite reductase that regulates responses to cellular hypoxia and reoxygenation. Figure 3.4 shows potential effects of NO on the pathophysiology of myocardial ischemia-reperfusion.

Whereas xanthine oxidoreductase (XOR) normally causes damage through the generation of ROS, NO is considered to protect against the damaging effects of myocardial ischemia-reperfusion injury. In the heart, inorganic nitrite ($NO_2^-$) has the potential to act as an endogenous store of NO, liberated specifically during ischemia. Under ischemic conditions, both rat and human homogenized myocardium and the isolated perfused rat heart generate NO from $NO_2^-$ in a reaction that depends on XOR activity and protects the myocardium from ischemia-reperfusion injury (Webb et al. 2004). Hence, if XOR is presented with $NO_2^-$ as an alternative substrate, the resultant effects of its activity may be protective, by means of its production of NO, rather than damaging.

Prostacyclin released by cardiac tissue in response to ischemia and reperfusion is derived, at least in part, from cyclooxygenase-2 (COX-2), which plays an important protective role in a setting of ischemia-reperfusion of the heart. This is evidenced by the detrimental effect of COX-2 inhibitors (celecoxib, meloxicam, DuP-697, and aspirin) in these conditions.

## Role of NO in Management of Cardiovascular Disorders

Cardiovascular mechanisms of action of various classes of NO-modulating agents have been reviewed in earlier sections. Cardiovascular disorders for which NO-based therapies are used are listed in Table 3.1. Cardioprotective effect of NO will be considered in this section.

### *Role of NO in Cardioprotection*

NO, either endogenous or exogenous, represents one of the most important defenses against myocardial ischemia-reperfusion injury. In the cardioprotective actions of NO, iNOS and mitochondria play an import role. The protective action of NO may be achieved through ischemic preconditioning, pharmacological cardioprotection, and gene therapy. The late phase of preconditioning is mediated by increased iNOS activity resulting in enhanced NO bioavailability. Various drugs (e.g., statins, ACE inhibitors, angiotensin-receptor blockers, etc.) also produce salubrious effects in experimental models of myocardial infarction via their enhancement of NO bioavailability. Thus, NO appears to be a common mediator of the protection afforded by a wide array of seemingly unrelated pharmacological and nonpharmacological interventions, underscoring its fundamental role as a ubiquitous defense of the heart against ischemia and reperfusion. This challenges the conventional wisdom that iNOS is deleterious during myocardial ischemia-reperfusion and instead proposes the concept that iNOS, when expressed in cardiac myocytes, is a profoundly protective protein. Although the precise molecular events remain to be defined, NO interacts with components of the electron transport chain and/or the mitochondrial permeability transition pore to limit postischemic myocardial damage, and this action potentially provides a fundamental molecular explanation for the mechanism of NO-mediated cardioprotection.

Ischemic reperfusion injury (IRI) occurs frequently in revascularization procedures such as coronary artery bypass graft (CABG). Conditioning of myocardial cells to an oxidative stress prior to IRI may limit the consequences of this injury. Preconditioning the myocardium with hyperbaric oxygen (HBO) before reperfusion has been shown to have a myocardial protective effect by limiting the infarct size

**Table 3.1** Cardiovascular disorders for which NO-based therapies are used

| |
|---|
| *Atherosclerosis* |
| *Coronary heart disease/myocardial ischemia* |
| Angina pectoris |
| Myocardial infarction |
| Adjunct therapy to coronary artery bypass graft (CABG) |
| Coronary artery restenosis |
| *Congestive heart failure* |
| *Hypertension* |
| *Hypercholesterolemia* |

postischemia and reperfusion. Current evidence suggests that HBO preconditioning may partly attenuate IRI by stimulating the endogenous production NO, which has the ability to reduce neutrophil sequestration, adhesion, and associated injury, and improve blood flow (Yogaratnam et al. 2008). HBO preconditioning-induced NO may play a role in providing myocardial protection during operations that involve an inevitable episode of IRI and protection of the myocardium from the effects of IRI during cardiac surgery.

Combined proteomic and metabolomic analyses provide direct evidence of the effect of protein expression on cellular processes, and this approach has been used to investigate the cardioprotective mechanisms following exposure to nitrate, which is an important bioactive molecule, capable of conferring cardioprotection and a variety of other benefits in the cardiovascular system (Perlman et al. 2009). Nitrate administration resulted in a short-lived increase in cardiac nitrate levels, but substantial elevations in cardiac ascorbate oxidation. This was accompanied by significant improvements in cardiac contractile recovery following ischemia-reperfusion after preconditioning with low or high nitrate doses. There was significant nitrite-induced protein modifications (including phosphorylation) revealed by MS-based proteomic studies. Altered proteins included those involved in metabolism (e.g., aldehyde dehydrogenase 2), redox regulation (e.g., protein disulfide isomerase A3), contractile function (e.g., filamin-C), and serine/threonine kinase signaling (e.g., protein kinase A R1α). Thus, brief elevations in plasma nitrite trigger a concerted cardioprotective response characterized by persistent changes in cardiac metabolism, redox stress, and alterations in myocardial signaling. These findings help elucidate possible mechanisms of nitrite-induced cardioprotection and have implications for nitrite dosing in therapeutic regimens. A similar mechanism may underpin the cardioprotective value of physical exercise and a diet containing nitrite/nitrate-rich foods.

## *Role of NO in the Management of Angina Pectoris*

Three common categories of drugs used for chronic stable angina pectoris are nitrates, beta-adrenoreceptor blockers and calcium channel blockers. Various forms of nitrates used are sublingual nitroglycerine, isosorbide dinitrate (slow release formulation), transdermal nitroglycerine, and oral isosorbide-5-monitrate. Other NO-based therapies have been investigated.

A new once-a-day formulation of molsidomine (16 mg) for patients with stable angina pectoris was evaluated in a pilot double-blind randomized placebo-controlled study (Messin et al. 2003). The pharmacokinetics of molsidomine with this dose in patients with stable angina pectoris was compatible with a once-daily dosage regimen. This formulation was found to be effective and well tolerated, providing a 24 h therapeutic control of myocardial ischemia. A positive and significant linear relationship between molsidomine plasma concentration and the increase in exercise tolerance was observed. Nitorol® (isosorbide dinitrate) Injection Syringe (Eisai Co Ltd) is the first nitric acid syringe formulation approved for the treatment of acute cardiac failure and unstable angina in Japan.

## *Role of NO in Therapy of Coronary Heart Disease*

Many studies have shown that loss of endothelium-derived NO is a major factor of ischemic episodes in patients with coronary artery disease and there is increasing evidence to suggest that nitric oxide might exert antiatherosclerotic actions. This is based on the results of animal studies showing the effects of lipid lowering drugs, antioxidants, angiotensin converting enzyme inhibitors, $Ca^{2+}$ channel blockers, estrogens, and agents which modulate NO bioavailability. These finds have been compared to the results of patient studies and clinical trials. In spite of encouraging results obtained with antioxidants in animals, clinical trials show a clear positive effect only of vitamin E treatment on the outcome of cardiovascular disease. Angiotensin-converting enzyme (ACE) inhibitors can ameliorate endothelial dysfunction in coronary heart disease, but their impact on disease progression remains unclear. There is evidence that estrogen replacement therapy in postmenopausal women may increase the bioavailability of NO. Finally, improved endothelial function and plaque stability clearly contribute to the clinical benefits of lipid lowering interventions, statins in particular. Taken together, these studies lend support to the concept that improving endothelial function and NO release might serve as valuable elements in the prevention or therapy of cardiovascular disease. Vasodilator action of NO has been used to facilitate the introduction of other therapeutics into coronary circulation.

In vivo studies in dogs have shown that contractile function in the ischemic myocardium resulting from coronary occlusion is significantly improved by nitroglycerin and L-nitro-arginine-methylester. The mechanism of metabolic modulation and myocardial protection activated by NO donors is by lowering of glucose uptake and lactate production. This is achieved by a reduction of translocation of cardiac glucose transporters via inhibition of activation of AMP-activated protein.

Intracoronary delivery of an adenovirus encoding murine adenylyl cyclase type VI (ACVI) was performed in adult pigs with and without simultaneous intracoronary infusion of NO donor nitroprusside (Roth et al. 2004). Addition of nitroprusside during intracoronary infusion of ACVI was associated with a fourfold increase in cAMP-generating capacity in the left ventricle. Intracoronary infusion of the nitroprusside is, therefore, a safe and effective means to increase the extent of cardiac gene transfer with intracoronary delivery of adenovirus vectors.

Oxidized LDL cholesterol and cytokines increase arginase and decrease NOS expression in human endothelial cells, leading to a decrease in NO production. In arteriosclerotic plaques, characterized by increased oxidized LDL and cytokine levels, a sustained local NO reduction enhances sensitivity of the downstream guanylyl cyclase system toward an acute NO increase. Application of the NOS substrate L-arginine or the NO donor isosorbide dinitrate preferentially dilates stenotic coronary artery segments increasing poststenotic coronary blood flow in patients with coronary artery disease without affecting the non-diseased arteries (Lauer et al. 2008).

## *NO-Releasing Aspirin in Patients Undergoing CABG*

One of the complications of coronary artery bypass (CABG) surgery is occlusion of the graft. This is likely to occur in venous graft in type 2 diabetes mellitus, which is known to negatively affect biological properties of venous vasculature, and, particularly, to reduce endothelium-derived NO release. A study has evaluated the functional effects of a NO-releasing aspirin on vein grafts of diabetics and control patients undergoing elective CABG (Lorusso et al. 2007). Significant impairment of endothelial-dependent vasodilation (acetylcholine induced) was documented in vein grafts of diabetic subjects. NO-releasing aspirin induced a significant and comparable vascular relaxation in all venous segments of diabetic patients. This preliminary study confirms the impairment of endothelium-dependent vasodilative property of vein grafts in diabetic patients and also showed that NO-releasing aspirin effectively induces vasodilation of vein grafts, which was effective also in diabetic patients thereby representing a promising adjunct therapy to CABG. Further studies are required to conclusively assess the safety and benefits of this pharmacological agent.

## *NO-Based Therapies for Congestive Heart Failure*

CHF results in cardiovascular dysfunction and diminished vascular NO production. In CHF, endothelial dysfunction may contribute to impairment of exercise-induced vasodilatation and decreased exercise capacity. In a randomized double-blind crossover study in patients with chronic stable CHF, oral supplementation with L-arginine prolonged exercise duration, which may be due to NO-induced peripheral vasodilatation (Bednarz et al. 2004).

CDP-1050 (Cordex Pharma) is designed to correct NO and redox imbalance in the failing heart and the cardiovascular system. The drug has a dual mechanism of action; it inhibits the production of tissue-damaging ROS, and restores NO to physiologic levels.

A randomized trial has examined whether a fixed dose of both isosorbide dinitrate and hydralazine (NitroMed's Bidil) provides additional benefit in blacks with advanced heart failure, a subgroup previously noted to have a favorable response to this therapy (Taylor et al. 2004). Hydralazine is an antioxidant and vasodilator, which means that it protects NO formed by isosorbide dinitrate and dilates blood vessels. Neither drug separately is indicated for heart failure. The addition of a fixed dose of isosorbide dinitrate plus hydralazine to standard therapy for heart failure including neurohormonal blockers was shown to be efficacious and increased survival among black patients with advanced heart failure. The study was terminated early owing to a significantly higher mortality rate in the placebo group than in the group treated with the drug combination. NitroMed Inc has submitted the African American Heart Failure Trial (A-HeFT) clinical dataset to the FDA. The product was approved by the FDA in 2005. In 2006 NitroMed announced data from

a continuing analysis of patients treated with BiDil®, which suggest that BiDil® decreases systolic blood pressure (SBP) in black heart failure patients with higher baseline SBP but not in those with lower baseline SBP. The beneficial effects of BiDil on clinical outcomes in A-HeFT were shown to be independent of baseline SBP. When differentiating patients by baseline SBP above or below the median (126 mmHg) in A-HeFT, the significant benefits demonstrated for BiDil on mortality and morbidity were similar for both groups.

## *NO-Based Therapy for Management of Cardiogenic Shock*

Cardiogenic shock (CS) is the leading cause of death among patients hospitalized with acute myocardial infarction (AMI). It is defined as tissue hypoperfusion resulting from ventricular pump failure in the presence of adequate intravascular volume. Rapid assessment and triage of patients presenting in CS followed by appropriate institution of supportive therapies including vasopressor and inotropic agents, mechanical ventilatory support, and intra-aortic balloon pump counterpulsation are usually employed in the management of these patients. However, emergency percutaneous coronary intervention or coronary artery bypass graft surgery may be required, but the mortality from CS, approximately 50%, remains high despite reperfusion therapy. There is a dire need for therapies to treat CS more effectively. One approach is based on the role of NO in CS as the balance of NO production and regulation of cardiac function is disrupted. Abnormal NO production inhibits cardiac contractility in failing myocardium.

Tilarginine, L-N-monomethyl arginine (L-NMMA) or N(G)-monomethyl-L-arginine HCL, a nonselective inhibitor of NOS, has been studied in clinical trials for the treatment of CS complicating myocardial infarction. Despite evidence that excessive NO production may contribute to the pathogenesis of CS complicating myocardial infarction, the results of these studies proved disappointing because of lack of benefit and excess mortality. Further studies using a selective iNOS inhibitor to reduce the pathological effects of excessive NO production while leaving the beneficial effects of vascular NO production by eNOS unaltered may prove to be of value (Howes and Brillante 2008).

## *NO-Based Therapy for Cardiac Arrhythmias*

Paroxysmal supraventricular tachycardia (PSVT), one of the most common cardiac arrhythmias, is a rapid, regular heart rate originating in the atria. Currently, adenosine is the only approved treatment for PSVT in the USA. Although both ATP and adenosine inhibit atrioventricular nodal conduction, ATP is believed to have dual inhibitory action; one mediated by adenosine, the product of its rapid enzymatic degradation, and the other a triggered vagal reflex. Vagal maneuvers aimed at

enhancing vagal tone to the heart, and thereby suppressing atrioventricular nodal conduction, have been clinically used to terminate tachycardia. Injectable formulations of ATP have been approved in Europe for over 50 years as safe and efficacious treatments for PSVT.

ATPace (intravenous ATP injection) is being developed by Cordex Pharma for the acute treatment and diagnosis of certain cardiac arrhythmias including the termination of PSVT, and is in phase III clinical trials in the USA. ATPace is also being developed to diagnose bradycardia, which is one of the main causes of fainting.

## *Prophylaxis of Cardiovascular Disorders*

Prevention is an important aspect of cardiovascular health. The value of physical exercise is well recognized. The role of NO in maintaining the integrity of the cardiovascular system is also established. Exercise increases the production of NO. But even with a regular exercise regimen, the body may not produce sufficient quantities of NO to maintain normal levels in unhealthy cells. This can be supplemented by natural sources of L-arginine including nuts, fruits, dairy, and meats as well as natural sources of L-citrulline including melon rinds and cucumbers. A low-fat diet also helps increase the body's production of NO. No also plays a role in retarding atherosclerosis with aging, an important risk factor for cardiovascular disease.

### Prevention of Atherosclerosis with Aging

Aging may contribute to the pathogenesis of atherosclerosis. Bioavailability of NO is limited in senescence, the effect of NO on senescence and its relationship to cardiovascular risk factors have been studied by investigating senescence-associated β-galactosidase (SA-β-gal) and human telomerase activity in human umbilical venous endothelial cells (HUVECs). Treatment with a NO donor, DETA-NO, and transfection with eNOS into HUVECs each decreased the number of SA-β-gal positive cells and increased telomerase activity (Hayashi et al. 2006). The NOS inhibitor L-NAME abolished the effect of eNOS transfection. The physiological concentration of 17β-estradiol activated hTERT, decreased SA-β-gal-positive cells, and caused cell proliferation. However, ICI 182780, an estrogen receptor-specific antagonist, and L-NAME each inhibited these effects. Treatment with L-arginine or L-citrulline of eNOS-transfected cells partially inhibited, and combination of L-arginine and L-citrulline with antioxidants strongly prevented high glucose-induced cellular senescence. These data demonstrate that NO can prevent endothelial senescence, thereby contributing to the antiaging action of estrogen. The ingestion of NO-boosting substances, including L-arginine, L-citrulline, and antioxidants, can delay endothelial senescence under high glucose. Prevention of endothelial senescence by NO and/or eNOS activation may have clinical utility in the treatment of atherosclerosis in the elderly.

## *Peripheral Vascular Disorders*

### Peripheral Atherosclerotic Arterial Disease

As mentioned earlier in the report, atherosclerosis is an inflammatory disease in which there is an increase in active inflammation markers such as C-reactive protein and other factors released by endothelial cells. Nitroglycerin acts by a chemical liberation of NO, which explains its anti-inflammatory action. In a randomized, double-blind, placebo-controlled pilot study, patients were treated with continuous application of a transdermal nitroglycerin patch (de Berrazueta et al. 2003). No biological parameter was modified in the placebo group but nitroglycerin significantly reduced plasma levels of C-reactive protein and sE-selectin and increased the levels of intraplatelet cGMP. The results show that nitroglycerin has an anti-inflammatory action in patients with peripheral vascular disease. This may provide a new therapeutic approach to understanding the efficacy of nitrovasodilators in the improvement of atherosclerotic syndromes.

### Peripheral Ischemic Disease

Lower-limb ischemia is a major health problem. Critical limb ischemia due to advanced peripheral arterial occlusive disease (PAOD) is characterized by reduced blood flow and oxygen delivery with exercise or even at rest with severe disease, resulting in claudication (muscle pain) and eventual non-healing skin ulcers that can lead to gangrene. The estimated incidence of critical limb ischemia is 500–1,000/million/year in the USA. Progressive microcirculatory dysfunction and impairment of angiogenesis/arteriogenesis are crucial pathophysiologic determinants of critical limb ischemia. As critical limb ischemia progresses, deregulation of the microcirculation occurs, characterized by activation of white blood cells, platelet aggregation, plugging of capillaries, endothelial damage, and release of free radicals, all of which promote further ischemia leading to tissue damage and eventual tissue necrosis. The prognosis of patients with critical limb ischemia is poor. The survival rate for patients with significant tissue necrosis without major amputation is less than 50% after 1 year. Many patients presenting with ischemic pain and ulcers are not suitable candidates for surgical revascularization or angioplasty due to diffuse, distal occlusive vascular disease. Current pharmacotherapy has had little impact on limb salvage in patients with advanced critical limb ischemia and, likewise, little symptomatic effect.

Because of the absence of effective treatment in the advanced stages of the disease, amputation is undertaken to alleviate unbearable symptoms. Novel therapeutic approaches include the intramuscular use of autologous bone marrow cells (BMCs). Because tissue ischemia is associated with an overwhelming generation of oxygen radicals and perturbed shear stress, metabolic intervention with antioxidants and L-arginine could potentially induce beneficial effects beyond those achieved by BMCs. The protective effect of autologous BMCs and vascular protection by metabolic cotreatment (vitamin E added to the chow and vitamin C

and L-arginine added to the drinking water) were examined in ischemia-induced angiogenesis in the mouse hind limb, a model of extensive acute peripheral arterial occlusion (Napoli et al. 2005). Intravenous BMC therapy improved blood flow and increased capillary densities and expression of Ki-67, a proliferation-associated protein. This beneficial effect was amplified by metabolic cotreatment, an intervention inducing vascular protection, at least in part, through the NO pathway, reduction of systemic oxidative stress, and macrophage activation. Therefore, although a cautious approach is mandatory when experimental findings are extended to human diseases, autologous BMCs together with metabolic intervention could be an effective clinical treatment for peripheral arterial disease.

### An eNOS Mutant as Therapeutic for Peripheral Vascular Ischemia

Nitric oxide (NO) plays an important role in angiogenesis by mediating some of the effects of vascular endothelial growth factor (VEGF) and other growth factors and by inhibiting local antiangiogenic mechanisms (e.g., VEGF receptor downregulation). In the setting of atherosclerotic arterial disease and the presence of multiple concurrent cardiovascular risk factors, activation of vascular endothelial cells leads to reduced production of eNOS and impaired local angiogenesis. A treatment that reestablishes a sufficient level of bioavailable NO can potentially lead to enhanced neovascularization and increased blood flow to an ischemic limb.

Genvascor (Cardium Therapeutics Inc), a preclinical, DNA-based, eNOS therapeutic is being designed to induce production of NO directed at mediating the effects of multiple growth factors to enhance neovascularization and increased blood flow for the treatment of patients with critical limb ischemia due to advanced peripheral arterial occlusive disease (PAOD). Based on its multiple vasculoprotective mechanisms, as well as the anti-inflammatory activity that NO exerts while also stimulating angiogenesis and arteriogenesis, treatment with Genvascor could lead to superior clinical efficacy to relieve peripheral limb ischemia over single growth factor treatments that are currently in development. The proprietary eNOS mutant has an increased specific activity of the NOS enzyme, which induces the production of high local levels of NO. This production is not only independent of the level of endogenous growth factors present, but also is not inhibited by common concurrent risk factors such as hypercholesterolemia or increased oxidative stress, which are known to inhibit the activity of endogenous wildtype eNOS.

### Sodium Nitrite Therapy for Peripheral Vascular Ischemia

Sodium nitrite may act as a site-specific NO donor via mechanisms regulated by tissue hypoxia and is preferable to administration of NO, which may be associated with problems of delivery and tissue specificity. Sodium nitrite therapy exerts cytoprotective effects against acute ischemia/reperfusion injury in both heart and liver, consistent with the model of bioactive NO formation from nitrite during ischemic stress. Chronic sodium nitrite therapy has been shown to selectively augment angiogenic activity and tissue perfusion in the murine hind limb ischemia

model (Kumar et al. 2008). Sodium nitrite significantly restored ischemic hind limb blood flow in a time-dependent manner, with low-dose sodium nitrite being most effective. Nitrite therapy significantly increased ischemic limb vascular density and stimulated endothelial cell proliferation. The effects of sodium nitrite therapy were evident within 3 days of the ischemic insult demonstrating the potency and efficacy of this therapy. Sodium nitrite therapy also increased ischemic tissue nitrite and NO metabolites compared to nonischemic limbs. Use of the NO scavenger carboxy PTIO completely abolished sodium nitrite-dependent ischemic tissue blood flow and angiogenic activity consistent with nitrite reduction to NO being the proangiogenic mechanism. These data demonstrate that chronic sodium nitrite therapy is an effective therapy for peripheral artery disease and critical limb ischemia.

### NO-Based Therapies for Raynaud's Phenomenon

Raynaud's phenomenon (RP) is characterized by transient reduction in blood supply through the small arteries in the hands and feet. RP is a cutaneous microvascular disorder whose pathophysiology appears to relate to disorders of local and/or reflex thermoregulatory control of the skin circulation. Severe RP can cause digital necrosis. It has been hypothesized that NO may have a role in RP. Oral L-arginine has been reported to reverse digital necrosis in RP suggesting that a defect in NOS metabolism is present in Raynaud's phenomenon. Thus there is a potential role for oral L-arginine therapy in Raynaud's phenomenon, especially in RP with digital necrosis.

Nitrates are often prescribed for the treatment of RP, but currently available formulations are limited by side effects, particularly headaches and dizziness. Symptoms improve by application of glyceryl trinitrate ointment. A novel formulation of topical nitroglycerin, MQX-503, was well tolerated and more effective than placebo for the treatment of RP in a randomized clinical trial (Chung et al. 2009).

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# Chapter 4
# Biomarkers of Cardiovascular Disorders

## Introduction

There are several definitions of biomarkers. A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of a physiological as well as a pathological process or pharmacological response to a therapeutic intervention. Classical biomarkers are measurable alterations in blood pressure, blood lactate levels following exercise and blood glucose in diabetes mellitus. Any specific molecular alteration of a cell on DNA, RNA, metabolite, or protein level can be referred to as a molecular biomarker. In the era of molecular biology, biomarkers usually mean molecular biomarkers and can be divided into three broad categories (Jain 2010):

1. Those that track disease progression over time and correlate with known clinical measures
2. Those that detect the effect of a drug
3. Those that serve as surrogate endpoints in clinical trials

While researchers are studying all three categories, biotechnology and pharmaceutical companies favor using biomarkers as drug discovery tools – not only to detect biological responses to experimental drugs but also to aid in the discovery of new targets for therapeutic intervention. A biomarker can be as simple as a laboratory test or as complex as a pattern of genes or proteins. From a practical point of view, the biomarker would specifically and sensitively reflect a disease state and could be used for diagnosis as well as for disease monitoring during and following therapy. The term "negative biomarker" is used for a marker that is deficient or absent in a disease.

Surrogate endpoint is a biomarker that is intended to serve as a substitute for a clinically meaningful endpoint and is expected to predict the effect of a therapeutic intervention. A clinical endpoint is a clinically meaningful measure of how a patient feels, functions, or survives. Clinical endpoints may be further classified as

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intermediate endpoints, which are clinical endpoints that are not the ultimate outcome, but are nonetheless of real clinical usefulness, for example, exacerbation rate and ultimate clinical outcomes, which are clinical endpoints reflective of the accumulation of irreversible morbidity and survival. These definitions indicate a clear hierarchical distinction between biomarkers and surrogate endpoints. While numerous laboratory biomarkers may be associated with a particular disease state, the term "surrogate" indicates the ability of a biomarker to provide information about the clinical prognosis or efficacy of a therapy. The word "surrogate" implies a strong correlation with a clinical endpoint, but in order to be clinically useful a surrogate must provide information about prognosis or therapeutic efficacy in a significantly shorter time than would be needed by following the clinical endpoint.

Historically, successful surrogates have linked effects on biomarkers for single effects in large populations but this framework needs to be expanded because it does not recognize multidimensional quality of clinical response and thus conflicts with current goals for individualized therapy. There is also the need to include possibility that multiple biomarkers may provide useful information in aggregate. A biomarker is valid if:

1. It can be measured in a test system with well-established performance characteristics.
2. Evidence for its clinical significance has been established.

## Biomarkers of Cardiovascular Diseases

The major use of cardiac biomarkers until very recently has been the detection of myocardial infarction (MI). The rationale of using the measurement of a protein in blood for this purpose is straightforward. The myocyte is the major cell in the heart, and the heart's purpose is to pump blood. Because myocytes essentially cannot be regenerated, if heart cells die, then cardiac function has a high probability of being impaired. When the cell dies, the proteins inside the cell will be released with proteins in the cytoplasm leaving the cell more rapidly than ones in membranes or fixed cell elements. The most sensitive markers should be those in highest abundance in the cell, and because the major function of the heart is contraction, the proteins involved in contraction and producing the energy to support it should be good candidates for biomarkers in blood. If such proteins have cardiac-specific forms, then specificity might be achievable as well as sensitivity. Two classical biomarkers for MI are serum creatine kinase and troponin. Elevated levels of C-reactive protein (CRP) are associated with increased risks of ischemic heart disease. One of the sequelae of MI is congestive heart failure (CHF) and biomarkers for this are also discussed. Systemic hypertension itself is a marker for heart disease and no separate markers are listed apart from genetic markers of hypertension. The relative risk of coronary heart disease is better predicted by two biomarkers than a single biomarker. More sophisticated multiplex panels have emerged from work with microarrays. A classification of biomarkers for cardiovascular diseases is shown in Table 4.1.

**Table 4.1** Classification of biomarkers for cardiovascular diseases

| |
|---|
| *Genetic biomarkers for heart disease* |
| IL-1 gene variations |
| Polymorphisms in the apolipoprotein E (APOE) gene |
| Mutations in the low density lipoprotein (LDL) receptor gene |
| Mutations within several genes that code for ion channel |
| Polymorphism in the angiotensinogen gene promoter |
| *Biomarkers for ischemic heart disease and acute myocardial infarction (AMI)* |
| Acute phase reactants: C reactive protein (CRP) |
| Biomarkers of myocardial dysfunction: natriuretic peptide |
| Biomarkers of myocardial infarction: troponins, creatine kinase (muscle brain), myoglobin, copeptin |
| Citric acid pathway metabolites |
| Copeptin |
| Cripto-1 |
| Fatty acid binding protein |
| Fetuin-A |
| High density lipoprotein 2 ($HDL_2$) |
| Inflammatory cytokines: IL-6 and TNF-α |
| Markers of ischemia: choline, ischemia-modified albumin, unbound free fatty acids |
| Plaque destabilization: intercellular adhesion molecules, vascular adhesion molecules, metalloproteinase-9 |
| Plaque rupture: Soluble CD40 ligand, placental growth factor |
| *Biomarkers for heart failure* |
| Angiogenesis biomarkers of coronary heart disease with asymptomatic left ventricle dysfunction |
| Beta-2a protein |
| Biomarkers of inflammation: galectin-3, C reactive protein, TNF-α, Fas (APO-1), IL-1, IL-6, IL-18 |
| Desmin |
| G protein-coupled receptor kinase-2 |
| MMP-CV2: microparticle protein biomarker of ischemic cardiac failure |
| Myocyte stress: natriuretic peptide, midregional fragment of proadrenomedullin, ST2 |
| Neurohormones: norepinephrine, renin, angiotensin II, aldosterone, arginine vasopressin, endothelin |
| Oxidative stress: oxidized LDLs, myeloperoxidase, urinary biopyrrins, plasma malondialdehyde |
| Urinary and plasma isoprostanes |
| *Biomarkers of risk factors for coronary heart disease* |
| Biomarkers of inflammation: C-reactive protein (CRP) and fibrinogen |
| Biomarkers of oxidative damage: endothelial progenitor cells (EPCs) |
| Plasma apolipoprotein AI and B levels |
| *Biomarkers for atherosclerosis* |
| Adipocyte enhancer-binding protein 1 |
| Asymmetric dimethylarginine (an endogenous nitric oxide synthaseinhibitor) |
| Cathepsin D |
| E-selectin |
| Ghrelin |
| HSP-27 (low levels) |
| Lipid-modified proteins |
| Lipoprotein-associated phospholipase A2 |
| Macrophages chemoattraction |
| Nitric oxide: impairment of production |
| Oxidative stress |
| Serum amyloid A |
| T-cell chemokine activity |
| *Imaging biomarkers of cardiovascular disease* |
| *Miscellaneous*: chromogranin, osteoprotegerin, adiponectin, growth differentiation factor 15, lens aging |

## Methods for Identification of Cardiovascular Biomarkers

### *Application of Proteomics for Biomarkers of Cardiovascular Disease*

Proteomics has opened new avenues in the search for clinically useful biomarkers of cardiovascular disease. As the number of proteins that can be detected in plasma or serum (the primary clinical diagnostic samples) increases toward 1,000, a paradoxical decline has occurred in the number of new protein markers approved for diagnostic use in clinical laboratories. MicroParticle Proteomics is developing technology for detection of protein microparticle biomarkers of cardiovascular diseases. Considering the limitations of current proteomics protein discovery platforms, an alternative approach is proposed that is applicable to a range of biological/physiological problems, in which quantitative mass spectrometric methods developed for analytical chemistry are employed to measure limited sets of candidate markers in large sets of clinical samples. A set of 177 candidate biomarker proteins with reported associations to cardiovascular disease have been presented as a starting point for such a "directed proteomics" approach (Anderson 2005).

Insilicos LLC is using pattern recognition with MS to PreCluedevelop cardiovascular disease biomarker panel as a clinical diagnostic for either screening or treatment-planning purposes. The future holds great promise for the availability of a panel of cardiac serum biomarkers able to delineate different stages of each heart disease, thus allowing the design of clinical interventions potentially using stage-specific therapeutics. All of this is feasible only with detailed information about the unique and selective protein modifications that occur during the development of heart disease. The combination of proteomic biomarkers with clinical phenotypes and genetic haplotype information can lead to a more precise diagnosis and therapy on an individual basis – personalized medicine (Arab et al. 2006).

### *Detection of Biomarkers of Myocardial Infarction in Saliva by a Nanobiochip*

The feasibility and utility of saliva as an alternative diagnostic fluid for identifying biomarkers of acute myocardial infarction (AMI) has been investigated. A lab-on-a-chip method was used to assay 21 proteins in serum and unstimulated whole saliva procured from AMI patients within 48 h of chest pain onset and from apparently healthy controls (Floriano et al. 2009). Both established and novel cardiac biomarkers demonstrated significant differences in concentrations between patients with AMI and controls. The saliva-based biomarker panel of CRP, myoglobin, and myeloperoxidase showed diagnostic capability, which was better than that of ECG alone. When used in conjunction with ECG, screening capacity for AMI was enhanced and was comparable to that of a panel of brain natriuretic peptide, troponin-I, creatine kinase-MB, and myoglobin. To translating these findings into

clinical practice, the whole saliva tests were adapted to a nanobiochip platform, which may provide a convenient and rapid screening method for cardiac events at point-of-care.

## *Metabolomic Technologies for Biomarkers of Myocardial Ischemia*

Recent advances in metabolic profiling technologies may offer the possibility of identifying novel biomarkers and pathways activated in myocardial ischemia. In one study, blood samples were obtained before and after exercise stress testing from patients who demonstrated inducible ischemia and those who did not and served as controls (Sabatine et al. 2005). Plasma was fractionated by liquid chromatography, and profiling of analytes was performed with a high-sensitivity electrospray triple-quadrupole mass spectrometer under selected reaction monitoring conditions. Lactic acid and metabolites involved in skeletal muscle AMP catabolism increased after exercise in both cases and controls. In contrast, there was significant discordant regulation of multiple metabolites that either increased or decreased in cases but remained unchanged in controls. Functional pathway trend analysis with the use of novel software revealed that six members of the citric acid pathway were among the 23 most changed metabolites in cases. Furthermore, changes in six metabolites, including citric acid, differentiated cases from controls with a high degree of accuracy. Application of metabolomics to acute myocardial ischemia has identified novel biomarkers of ischemia, and pathway trend analysis has revealed coordinate changes in groups of functionally related metabolites.

Proton nuclear magnetic resonance ($^1$H NMR) spectra of human serum can diagnose and determine the severity of coronary artery disease. It can provide an accurate and rapid diagnosis of coronary artery disease and be used for screening as well as for effective targeting of treatments such as statins.

## *Imaging Biomarkers of Cardiovascular Disease*

Molecular imaging techniques reveal processes at the molecular level, for example, expression or activity of a biomarker or the activity of a biological pathway. Molecular targets that are currently being investigated experimentally by molecular imaging probes in vivo have an important role in the development of atherosclerosis and acute plaque rupture, as well as in myocardial disease (Shaw 2009). Molecular imaging technology is now being translated into clinical application in humans for diagnosis, risk assessment, and response to treatment. Imaging-based surrogate biomarker end points could facilitate the development of new drugs, particularly those with novel mechanisms of action. Molecular imaging, by revealing multiple biomarkers and pathways function in vivo, will contribute to an understanding of cardiovascular disease biology at systems-level.

### Annexin A5 as an Imaging Biomarker of Cardiovascular Disease

Annexin A5, a plasma protein, has strong affinity for phosphatidylserine (PS) – a plasma cell membrane phospholipid. Coupling of Annexin A5 to contrast agents enables in vivo visualization of apoptotic cell death, which is manifested by externalization of PS (Laufer et al. 2008). These imaging studies have provided novel insight into the extent and kinetics of apoptosis in cardiovascular disease. Furthermore, Annexin A5 imaging has proven to be a suitable imaging biomarker for the evaluation of apoptosis-modifying compounds and plaque stabilizing strategies. Annexin A5 not only binds to exteriorized PS, but is also internalized through an Annexin A5-specific mechanism indicating that Annexin A5 imaging can also be used to visualize inflammation and cell stress. This will enable a better understanding of pathological processes underlying cardiovascular diseases.

### Cardiovascular MRI

Cardiovascular MRI has recently emerged as a powerful tool for detecting cardiovascular biomarkers and has an important role, particularly in visualizing several reversible and irreversible myocardial tissue changes (Schulz-Menger and Abdel-Aty 2008). It has potential applications in nonischemic and inflammatory cardiomyopathies. Cardiac MRI is helpful in making a differential diagnosis between ischemic and dilated cardiomyopathy, identifying patients with myocarditis, diagnosing cardiac involvement in sarcoidosis and Chagas' disease, identifying patients with unusual forms of hypertrophic cardiomyopathy, and defining the sequelae of ablation treatment for hypertrophic obstructive cardiomyopathy (Sechtem et al. 2007).

### Myocardial Perfusion Imaging

Myocardial perfusion imaging (MPI) is used as a primary screen to identify the presence of coronary artery disease (CAD) as evidenced by detection of areas of poor blood flow in the heart that can be caused by the formation of plaques that block the normal flow of blood to the heart. A pharmacologic stress agent is used to increase blood flow through coronary arteries temporarily during stress testing in order to more strikingly define areas of the heart that receive poor blood flow. The adenosine A2A receptor is the receptor subtype responsible for coronary vasodilation, or the widening of blood vessels.

Stedivaze (apadenoson) is a potent agonist of the A2A and offers improved selectivity over other adenosine receptor subtypes (A1 and A2B). Phase II studies suggest that Stedivaze produces ample coronary vasodilatory activity needed for cardiac stress MPI and has a pharmacokinetic profile that will allow it to be administered as a fixed dose bolus injection. Because of its improved selectivity for the A2A receptor subtype and its optimal pharmacokinetic profile, Stedivaze may offer improved tolerability over other adenosine receptor agonists currently marketed for use in pharmacologic stress MPI. Stedivaze is in phase III clinical development by Clinical Data Inc for use as a pharmacologic agent for MPI with the goals of demonstrating equal efficacy and improved tolerability compared to adenosine.

Over 7.6 million MPI tests were performed in the USA in 2008 and ~3.5 million of these tests required the use of a pharmacological agent to generate maximum coronary blood flow in lieu of exercise. With increasing aging population, there is a rise in the number of patients unable to perform exercise during diagnostic procedures, and emerging imaging modalities that require the use of a vasodilator as a biomarker.

## Applications of Biomarkers of Cardiovascular Disease

Novel biomarkers have improved diagnosis and prediction of outcome in AMI, but only troponin has been used to direct therapeutic intervention and none of the new prognostic biomarkers have been tested and proven to alter outcome of therapeutic intervention. Randomized trials are urgently needed to address this translational gap before the use of novel biomarkers becomes common practice to facilitate personalized management following an acute coronary event (Chan and Ng 2010).

### *Biomarkers for Ischemic Heart Disease and Myocardial Infarction*

Evaluation of patients who present to the hospital with a complaint of chest pain or other signs or symptoms suggestive of acute coronary syndrome is time consuming, expensive, and problematic. Recent investigations have indicated that increases in biomarkers upstream from biomarkers of necrosis (cardiac troponins I and T), such as inflammatory cytokines, cellular adhesion molecules, acute-phase reactants, plaque destabilization and rupture biomarkers, biomarkers of ischemia, and biomarkers of myocardial stretch may provide earlier assessment of overall patient risk and aid in identifying patients with higher risk of an adverse event. Only two categories of the biomarkers are approved – troponins and natriuretic peptide. Specifications that have been addressed for these biomarkers will need to be addressed with the same scrutiny for the newer biomarkers under investigation. These include validating analytical imprecision and detection limits, calibrator characterization, assay specificity and standardization, pre-analytical issues, and appropriate reference interval studies. Crossing boundaries from research to clinical application will require replication in multiple settings and experimental evidence supporting a pathophysiologic role and, ideally, interventional trials demonstrating that monitoring single or multiple biomarkers improve outcomes.

#### Troponin

The contractile unit (or sarcomere) of striated muscle fiber is composed of thick and thin filaments. The thick filament is composed mainly of myosin. Actin, tropomyosin, and troponin comprise the thin filament. Muscle contraction occurs when the thick and

thin filaments slide past each other, thereby shortening the length of the sarcomere. The interaction between the thick and thin filaments is regulated by the troponin complex found on the thin filaments. The troponin complex is composed of three protein subunits: troponin-I (TnI), troponin-T (TnT), and troponin-C (TnC). The calcium-mediated contraction of striated muscle (fast-skeletal, slow-skeletal, and cardiac muscle) is regulated by the troponin complex; contraction of smooth muscle is regulated by calmodulin. Three distinct tissue-specific isoforms of TnI have been identified: two in skeletal muscle and one in cardiac muscle, cTnI, which has never been isolated from skeletal muscle. Within the heart, cTnI appears to be uniformly distributed throughout the atria and ventricles. This absolute specificity of cTnI for cardiac tissue makes it an ideal biomarker of myocardial injury. It is released into the circulation when myocardial injury occurs and has become the basis for a standard test in combination with clinical and electrocardiographic findings for physicians to conduct prompt and effective triage of patients presenting with chest pain. Since the FDA first cleared an assay for cardiac troponin testing in 1996, the number of cTnI and cTnT assays has increased to over 20, in both quantitative and qualitative formats, using both central laboratory and point-of-care testing platforms.

A chemiluminescent microparticle immunoassay for the quantitative determination of cardiac troponin I in human serum and plasma on an automated immunoassay instrument system (ARCHITECT) can be used to aid in the diagnosis of myocardial infarction. The role of cardiac troponin point-of-care testing for predicting adverse outcomes in acute coronary syndrome patients has been investigated using i-STAT cTnI assay and found to be useful for risk stratification (Apple et al. 2006).

Cardiac troponin monitoring for detection of myocardial injury has been designated the new standard for differentiating the diagnosis of unstable angina and non-ST-elevation myocardial infarction in acute coronary syndrome patients. Increased cardiac troponin I (cTnI) or T (cTnT) in the clinical setting of ischemia is defined as an acute myocardial infarction and has been endorsed by the European Society of Cardiology, American College of Cardiology, and the American Heart Association.

The clinical significance of increased concentrations of cardiac troponins observed in patients with renal failure in the absence of acute coronary syndrome is controversial. One study has shown that the form of troponin observed in serum of renal failure patients is predominantly the free intact form, as in patients with acute coronary syndrome and is considered to reflect cardiac pathology (Fahie-Wilson et al. 2006).

In 2008, Compugen granted an option to Biosite to CGEN-144, a variant of troponin I biomarker, which it had discovered and verified to be differentially expressed as serum protein in myocardial infarction patients compared to healthy individuals. It was predicted by its immunoassay biomarker computational discovery platform. Biosite developed and selected antibodies that bind to the molecule to determine assay sensitivity and specificity in various disease states and as an addition to the currently commercialized troponin I test.

A multicenter study tested the diagnostic accuracy of 4 sensitive cardiac troponin assays (Abbott's Architect Troponin I, Roche High-Sensitive Troponin T,

Roche Troponin I, and Siemens' Troponin I Ultra) as well as a standard assay (Roche Troponin T) that were performed on blood samples obtained in the emergency department from patients who presented with symptoms suggestive of acute MI (Reichlin et al. 2009). The diagnostic performance of sensitive cardiac troponin assays was excellent, and these assays can substantially improve the early diagnosis of acute MI, particularly in patients with a recent onset of chest pain. Another multicenter study determined levels of troponin I as assessed by High-Sensitive Troponin T and traditional biomarkers of myocardial necrosis in patients with suspected acute MI, on admission and 3 and 6 h after admission (Keller et al. 2009). It concluded that the use of a sensitive assay for troponin I improves early diagnosis of acute MI and risk stratification, regardless of the time of onset of chest-pain.

In most patients with stable coronary artery disease, plasma cardiac troponin T levels are below the limit of detection for the conventional assay. A study with follow-up period of >5 years used a new, high-sensitivity assay to determine the concentration of cardiac troponin T in plasma samples from patients with stable coronary artery disease and analyzed the results of the assay in relation to the incidence of cardiovascular events (Omland et al. 2009). After adjustment for other independent prognostic indicators, cardiac troponin T concentrations as measured with a highly sensitive assay were significantly associated with the incidence of cardiovascular death and heart failure but not with MI in patients with stable coronary artery disease.

### Natriuretic Peptide

Several terms relevant to natriuretic peptide (NP) are defined as follows:

- B-Type Natriuretic Peptide (BNP) is a biologically active hormone, which corresponds to the C terminal fragment of proBNP (amino acids 77–108) and is present in both myocardium and plasma.
- Pre-proBNP is the cellular precursor synthesized in the myocardial cell. It contains 134 amino acids, including a signal peptide of 26 amino acids and is present only in myocardial tissue.
- ProBNP contains 108 amino acids (1–108) and is produced from pre-proBNP by cleavage of the signal peptide, when appropriate signals for hormone release are given. It is present in both myocardium and plasma.
- NT-proBNP, the entire N-terminal fragment of proBNP (amino acids 1–76), lacks hormonal activity and is present in both myocardium and plasma. Further degradation products of this molecule are sometimes identified with the same abbreviation, for example, NTproBNP (amino acids 1–21), but little metabolic and pathophysiologic information is available for these molecules.

The NP system is primarily an endocrine system that maintains fluid and pressure homeostasis by modulating cardiac and renal function. The physiologic functions of the NP system in healthy humans and in patients with cardiovascular disease are

not fully understood. NP levels are elevated in patients with CHF and other cardiac diseases; measurement of NPs may be used in the clinical setting to aid diagnosis and prognosis. In addition, synthetic NPs such as nesiritide are available for use in management of patients with acutely decompensated CHF. Not only do NPs modulate volume and pressure homeostasis, but they also exert important antiproliferative, antifibrotic effects in the heart. Thus, NPs may prove useful for prevention of remodeling after myocardial infarction and in advanced CHF. BNP is emerging as an important biomarker in patients with CHF and other cardiovascular diseases, such as pulmonary hypertension and atherosclerotic vascular disease. Elevated NP levels may serve as an early warning system to help to identify patients at high risk for cardiac events. Recombinant human ANP (carperitide) and BNP (nesiritide) are useful for management of acutely decompensated HF; these drugs are also being investigated for myocardial and renal protection in the setting of cardiac surgery and for prevention of cardiac remodeling. The clinical application of NPs is expanding rapidly. Recent basic science and clinical research findings continue to improve our understanding of the NP system and guide use of ANP and BNP as biomarkers and as therapeutic agents (Silver 2006).

Because BNP and NT-proBNP are released mainly from the cardiac ventricles in response to increased stretch and wall tension, it is not surprising that increased plasma concentrations of these NPs have been described in CHF, asymptomatic left ventricular dysfunction, arterial and pulmonary hypertension, cardiac hypertrophy, valvular heart disease, arrhythmia, and acute coronary syndrome. Because BNP and NT-proBNP are increased in a variety of cardiac and noncardiac diseases, caution must be exercised in interpreting the results in the context of clinical situation. BNP and NT-proBNP may become components of a panel of biomarkers, along with cardiac troponin, to be used for risk stratification in acute coronary syndrome. Commercially available BNP/NT-proBNP assays are:

- AxSYM BNP (Abbott)
- Centaur BNP (Bayer)
- Triage BNP (Biosit)
- IRMA BNP (Shionogi)
- StatusFirst CHF NT-proBNP (Nanogen, licensed from Roche)

StatusFirst CHF NT-proBNP rapid test is CE-marked and has been cleared by the FDA for diagnostic use with EDTA plasma samples. It measures circulating levels of NT-proBNP and provides a quantitative assessment of the biomarker's concentration in as few as 15 min via a small, low-cost reader. The test and reader are designed for use in emergency rooms and hospital laboratories.

### Copeptin

Copeptin, the C-terminal part of the vasopressin prohormone, is secreted stoichiometrically with vasopressin. The vasopressin system is activated after AMI.

Copeptin may predict adverse outcome, especially in those with an elevated NTproBNP (Khan et al. 2007). An assay based on C-Terminal Provasopressin (Copeptin), a patented biomarker for AMI, was introduced by Thermo Fisher Scientific in Europe in 2009 with plans to introduce it in the USA in 2010. In combination with a troponin biomarker test, the copeptin assay enables physicians to rule out or confirm the onset of myocardial infarction within minutes. Copeptin is also a biomarker for prognosis of intracerebral hemorrhage.

### Creatine Kinase Muscle Brain

Creatine kinase muscle brain (CK-MB), an enzyme that is involved in muscle metabolism, is found in both heart muscle tissue and skeletal muscle, albeit at a much lower concentration in the latter. CK-MB is released into the blood stream when cardiac muscle is damaged, hits a plateau where its levels are highest, and then eventually returns to lower levels. Although CK-MB analysis is currently the benchmark for biomarker detection of MI, similar patterns of CK-MB release can be caused by renal kidney failure, skeletal muscle trauma, and other unrelated ailments.

### Myoglobin

Myoglobin is a heme protein found in the cytosol of both cardiac and skeletal muscle. Following the death of cardiac tissue, myoglobin is the first cardiac marker to increase above normal levels in the blood. Myoglobin measurements have proven useful as an early marker for heart attack but other noncardiac-related trauma can also cause circulating levels of myoglobin to increase, so it cannot be used exclusively.

### Fatty Acid Binding Protein

Recent data suggest that serum-free fatty acid concentrations increase well before markers of cardiac necrosis and are sensitive indicators of ischemia in MI. Fatty acid binding protein (FABP) is abundant in cardiac muscle and is presumed to be involved in myocardial lipid homeostasis. Similar to myoglobin, plasma FABP increases within 3 h after onset of MI and returns to reference values within 12–24 h (Azzazy et al. 2006). FABP is useful for detecting cardiac injury in acute coronary syndromes and assays are available for this biomarker. Used with evidence investigator™ biochip system (Randolph Laboratories), FABP is the best early marker of MI with a sensitivity superior to either myoglobin and troponin. In collaboration with St James's hospital in Dublin, blood samples of chest pain patients were taken over a period of months and a subset of the samples analyzed for novel and routine biomarkers. Post pain onset was the best time point for diagnosis and FABP levels at 4–7 h had better sensitivity than any other single cardiac marker. Further analyses revealed that a combination of FABP, troponin, and CK-MB accurately identified

all the MI patients in the study cohort, demonstrating 100% sensitivity for the condition. Five non-MI patients had elevated FABP levels, three of which decreased after 1 h and the two others remained elevated. On further investigation these two patients had serious cardiac complications and previous MIs.

### High Density Lipoprotein 2

Alterations in protein composition and oxidative damage of high density lipoprotein (HDL) impair the cardioprotective properties of HDL. $HDL_2$ of subjects with CAD carries a distinct protein cargo and protein oxidation helps to generate dysfunctional HDL. Targeted tandem MS analysis of the model's significant features has revealed that $HDL_2$ of CAD subjects contained oxidized methionine residues of apolipoprotein A-I and elevated levels of apolipoprotein C-III. A proteomic signature composed of MALDI-MS signals from apoA-I, apoC-III, Lpa, and apoC-I accurately differentiated between CAD and control subjects (Vaisar et al. 2010). Thus models based on selected identified peptides in MALDI-TOF MS of the HDL may have diagnostic potential. Based on this, Insilicos LLC is developing PreClue cardiovascular disease biomarker panel as a clinical diagnostic.

### Cripto-1 as a Biomarker of Myocardial Infarction

Cripto-1, a member of the EGF-related proteins, is implicated in carcinogenesis and is also a biomarker for infarcted cardiac tissues. It is overexpressed in infarcted myocardial tissue, and not expressed or weakly expressed in non-infarct-related heart disease tissues and normal tissues. Furthermore, the overexpression of Cripto-1 correlates with the hypoxia-inducible factor-1-alpha indicating specificity to ischemic heart tissue. The expression of Cripto-1 has also been shown to be highly expressed in stem cells, which may have an important role in the repair of damaged myocardial tissue. This could represent a new biomarker for the diagnosis of myocardial infarction as well as a surrogate biomarker to monitor the healing process including regenerative stem cell activity of the infarcted myocardial tissue.

### Cataract as a Biomarker of Ischemic Heart Disease

There is an association between aging of the lens and ischemic heart disease (IHD), which may well be a causative one. Both lens proteins, plasma proteins, and the collagen of the vascular walls are subject to denaturation by a spontaneous reaction between aminogroups and reducing sugars, glycotoxins from tobacco smoke, or lipid peroxidation products. The spontaneous reaction leads to formation of advanced glycation end products (AGEs), which have been shown to increase atherogenicity of LDL-particles as well as playing a role in stiffening of arterial vessel walls both of which are important features in the pathogenesis of IHD. In the lens of the human eye, AGEs lead to accumulation of yellow, fluorescent compounds, thus causing the intrinsic fluorescence.

Cataract is a biomarker of the risk of and/or presence of cardiovascular disease, but the grading of cataract and its subclinical precursors is generally subjective and crude. Assessment of age-related changes in the lens could be used as a biomarker for IHD. A study was conducted to examine if the risk of IHD could be estimated by fluorophotometric assessment of lens aging (Kessel et al. 2006). This study compared lens fluorometry with an established method of prediction of IHD in healthy individuals. Lens fluorescence was measured on the undilated eye using a noninvasive ocular fluorophotometer, the Fluorotron (OcuMetrics, Mountain View, CA). Excitation was from 430 to 490 nm and detection from 530 to 630 nm. Presence and severity of lens opacities was determined from retroilluminated lens photographs focused on the anterior lens surface taken after dilation of the pupil using a Canon 60UVi camera (Canon Inc, Japan). The degree of cortical lens opacities was graded. The results of this study suggest that lens fluorescence can be used as an indicator of tissue damage caused by advanced glycation end products and that lens fluorescence can be used to give an estimate of the risk of IHD related to the effect of advanced glycation end products.

#### Plasma CD93 as a Biomarker for Coronary Artery Disease

A common SNP in the CD93 gene has been associated with risk of CAD. CD93 is a transmembrane glycoprotein, which is detectable in soluble form in human plasma. The results of a case-control study of premature MI and a nested case-control analysis of a longitudinal cohort study of 60-year-old subjects show that increased concentration of soluble CD93 in plasma is a biomarker for CAD, including MI (Mälarstig et al. 2011).

#### Plasma Fetuin-A Levels and the Risk of Myocardial Infarction

Fetuin-A, a protein almost exclusively secreted by the liver, induces insulin resistance and subclinical inflammation in rodents. Circulating fetuin-A levels are elevated in humans with metabolic syndrome and insulin resistance, conditions that are associated with increased risk of cardiovascular disease. A case-cohort study based on the European Prospective Investigation into Cancer and Nutrition-Potsdam Study has shown a link between high plasma fetuin-A levels and an increased risk of MI (Weikert et al. 2008).

## *Biomarkers of Congestive Heart Failure*

CHF, which occurs when an impaired heart muscle cannot pump blood efficiently, is a growing health problem and major cause of cardiac death. The diagnosis of heart failure may be challenging because its symptoms can overlap those of other conditions. Missing a heart failure diagnosis can put patients at high risk of serious problems, including death, but overdiagnosis may lead patients to receive unnecessary

treatment. Several biomarkers of CHF overlap with those of other cardiovascular disorders as several pathologies are in operation when the heart fails. Some of these biomarkers have already been discussed under other disease categories.

### Angiogenesis Biomarkers

Patients with CAD and left ventricular systolic dysfunction (LVSD) are often asymptomatic. Angiogenesis is implicated in the physiology of vascular repair and cardiac remodeling, and is one of many pathophysiological processes implicated in heart failure. Plasma indices associated with angiogenesis – angiogenin, vascular endothelial growth factor (VEGF), and angiopoietin (Ang)-1 and Ang-2 – are hypothesized to be abnormal in CAD patients with LVSD, being correlated with EF and wall motion abnormalities wall motion score independently of underlying coronary atheroma score. However, study results show that levels of angiogenin are inversely related with EF and positively with coronary atheroma scores, but not independently of EF (Patel et al. 2009). Other angiogenic markers were unrelated to objective measures of LVSD but VEGF were lower amongst those patients with heart failure. Angiogenin levels were related to wall motion scores. Thus, heart failure has only a modest impact on biomarkers of angiogenesis, in patients with CAD. Further research is warranted into the diagnostic and prognostic utility of biomarkers of angiogenesis for detection of asymptomatic ventricular dysfunction.

### Beta-2a Protein as a Biomarker of Heart Failure

Increased activity of single ventricular L-type $Ca^{2+}$ channels (L-VDCC) is a hallmark in human heart failure. Calcium plays a key role in controlling heart beat and this calcium ion channel is regulated by a group of accessory proteins, a major component of which is the beta-2a regulatory protein. In many types of terminal human heart failure beta-2a protein is increased or overexpressed, and the electrical activity in the heart's cells show a distinct pattern of single-channel activity, an indicator that the influx of calcium into the heart has also increased (Hullin et al. 2007). In a mouse model with an overexpressed beta-2a protein, the heart received a sustained increase of calcium, and slowly became hypertrophied and eventually failed. All the changes in the animal model – biochemical, physiological, and pathological – resembled the human heart failure process. This finding shows the potential values of beta-2a as a biomarker of heart failure and provides the therapeutic possibility to lower beta-2a in a heart that is beginning to fail.

### Desmin

Desmin is one of the fundamental cytoskeleton proteins of cardiomyocytes, and has a mechanical, structural, and regulatory function. In comparison with healthy individuals, patients with heart failure (HF) present different expression of desmin

content, that can be associated with its abnormal structure, different distribution, localization and disturbed function. Abnormal expression of desmin in cardiomyocytes plays a key role in progression of HF. Desmin content in cardiomyocytes, obtained by endomyocardial biopsy, correlates with long-term prognosis in HF patients (Pawlak et al. 2009). The low expression of desmin in cardiomyocytes with immunohistochemical assay is associated with unfavorable clinical course.

Desmin is a tissue biomarker and requires an invasive procedure for biopsy of heart muscle. Desmin is also linked to other diseases such as Alzheimer's disease and cancer. However, serum desmin is being investigated as a biomarker of colorectal cancer.

### Galectin-3 as Biomarker of Acute Heart Failure

A collaborative study by researchers from Massachusetts General Hospital (MGH) and the University Hospital of Maastricht, The Netherlands, has identified a new candidate biomarker for heart failure with the potential of further improving the challenging task of diagnosing and predicting outcomes for patients with symptoms of heart failure, primarily shortness of breath. Elevated blood levels of galectin-3, an inflammatory protein, can help diagnose heart failure and identify patients at risk of dying within 60 days (van Kimmenade et al. 2006). Another potential marker, apelin, did not prove to be useful in a comparative study. The combination of galectin-3 with NT-proBNP was the best predictor for prognosis in subjects with acute HF. The strong predictive power of galectin-3 indicates that heart failure is also an inflammatory process. The role of galectin-3 in heart failure may lead to new standards for therapeutic decision making or development of new agents that would inhibit this inflammatory cascade. The authors of this study are going to be using the PRIDE study data (see preceding section) to examine several other candidate markers that could lead to development of a comprehensive biomarker profile to guide targeted application of specific therapies.

### G Protein-Coupled Receptor Kinase-2 as Biomarker of CHF

The enzyme GPCR kinase-2 (GRK2 or β-ARK1) regulates β-adrenergic receptors (β-ARs) in the heart, and its cardiac expression is elevated in CHF. It is a potential biomarker for CHF. Decreasing or inhibiting the enzyme reverses heart failure in animal experiments. GRK activity participates with β-AR density to regulate catecholamine-sensitive cAMP responses. Myocardial GRK2 expression and activity are mirrored by lymphocyte levels of this kinase, and its elevation in CHF is associated with the loss of β-AR responsiveness and appears to increase with disease severity (Iaccarino et al. 2005). Therefore, lymphocytes may provide a surrogate for monitoring cardiac GRK2 in human CHF. There are plans to perform large human trials to specifically look at levels of GRK2 to see if they can predict responses to drugs such as β-blockers or other treatments for heart failure. It will also help to determine if GRK2 is a biomarker that can predict response to various therapies. If a drug lowers GRK2 levels, it should help the patient with CHF.

In CHF, the β-adrenergic receptor system fails to work properly. One of the functions of GRK2 is to turn off β-ARs.

GRK2 has now been shown to lead to permanent damage after myocardial infarction. Over production of GRK2 following a heart attack actually stimulates pro-death pathways in myocytes outside of the initial zone of damage. There is an inverse link between GRK2 activity and the production of NO, a molecular messenger that protects the heart against damage caused by a sudden loss of blood. When there is more GRK2, there is less NO, and vice versa. GRK2 may be affecting NO production by inhibiting the prosurvival protein kinase Akt, which itself regulates NO. These conclusions are based on a study that used gene therapy to inhibit GRK2, and found heart muscle cells in mice were substantially protected against destruction that would otherwise occur after an induced myocardial infarction (Brinks et al. 2010). Conversely, mice engineered to express excess GRK2 had more damage than would have been expected after myocardial infarction. These findings suggest that humans experiencing a heart attack might be helped with prompt delivery of a therapeutic targeting inhibition of GRK2. While it may be years before this concept can be tested in patients experiencing myocardial infarction, anti-GRK2 gene therapy could be tested in patients with CHF much sooner. A phase I clinical trial for GRK2-targeted gene therapy is preparing to be launched, pending FDA approval.

### KIF6 Gene as Biomarker of CHF

Carriers of the KIF6 (kinesin family member 6) wild-type gene are 50–55% more likely to develop CHF. KIF6 as a biomarker of CHF is the basis of a genetic test, StatinCheck, developed by Celera and offered through Berkeley HeartLab, which is owned by Celera. It is now licensed by clinical laboratory of Aurora Health Care in Milwaukee, Wisconsin.

Physicians can use the KIF6 test to identify the increased risk for CHF and begin treating their patients with statins. A study investigated whether 35 genetic polymorphisms, previously found to be associated with cardiovascular disease, were associated with MI in the CARE (Cholesterol and Recurrent Events) trial and with coronary heart disease (CHD) in the WOSCOPS (West of Scotland Coronary Prevention Study). In both the CARE and the WOSCOPS trials, carriers of the KIF6 719Arg allele had an increased risk of coronary events, and pravastatin treatment substantially reduced that risk (Iakoubova et al. 2008). Carriers of the 719Arg allele of KIF6 have 34% higher risk of MI and 24% higher risk of CHD compared with noncarriers among 25,283 women from the Women's Health Study, confirming and extending previous reports (Shiffman et al. 2008).

### NT ProBNP as Biomarker of CHF

The PRIDE study showed NT-proBNP to be highly sensitive and specific for the diagnosis of CHF in patients with shortness of breath and to strongly predict patient

deaths (Baggish et al. 2006). Furthermore, a multicenter, international study showed that NT-proBNP testing was valuable for diagnostic evaluation and short-term prognosis estimation in patients with shortness of breath due to suspected or confirmed acute CHF and should establish broader standards for use of the NT-proBNP in such patients (Januzzi et al. 2006). A major concern about the widespread use of the marker had been previous assertions that kidney disease, which is very common in patients with heart failure, might confound the results of NT-proBNP testing, since levels of the marker were higher among those with reduced renal function. A large-scale analysis has shown that NT-proBNP blood test previously found useful in diagnosing or ruling out heart failure in emergency room patients remains effective in patients with chronic kidney disease (Anwaruddin et al. 2006). The study also demonstrates that it can identify patients at a higher risk for death, independent of kidney dysfunction. Besides the diagnostic value of NT-proBNP, it was an even stronger predictor of death in breathless patients with significant renal insufficiency, emphasizing the fact that the marker is likely detecting a true signal of cardiac disease in these patients. This is a big step forward in the understanding of the optimal application of NT-proBNP measurement, as it removes one of the biggest obstacles that remained for the marker.

### Oxidative Stress as Biomarker of Heart Failure

Nanosensing techniques have been developed to monitor the physiology of NO in the beating heart in vivo. These methods involve the application of nanosensors to monitor real-time dynamics of NO production in the heart as well as the dynamics of oxidative species (oxidative stress) produced in the failing heart (Malinski 2005). Results of a recent study using nanotechnology demonstrated that African Americans have an inherent imbalance of NO, $O_2$-, and ONOO- production in the endothelium. The overproduction of $O_2$- and ONOO- triggers the release of aggressive radicals and damages cardiac muscle (necrosis), which may explain why African Americans are at greater risk for developing cardiovascular diseases, such as hypertension and heart failure, and are more likely to have complications than European Americans. Potential therapeutic strategies to prevent or ameliorate damage to the heart during cardiac events are prevention of $O_2$- and ONOO- production, supplementation of NO (NO donors), and scavenging of $O_2$- (antioxidants).

### Future Prospects for Biomarkers of Heart Failure

A biomarker profile may be a valuable addition to the conventional classification of heart failure according to underlying pathological conditions. Rather than focus on an individual biomarker, an approach involving multiple biomarkers is useful for refining risk stratification among patients with acute coronary syndromes. For example, the use of data on BNP together with troponin has been shown to achieve better risk stratification than that obtained with either biomarker alone. The accuracy of risk prediction is enhanced when a natriuretic peptide is coupled with other biomarkers of myocardial stress or inflammatory biomarkers. The next step might

be to obtain a profile by measuring representatives of distinct classes of biomarkers. The use of biomarkers for targeted therapy and for monitoring therapy requires extra effort, which would be worthwhile as it would likely enhance their clinical value (Braunwald 2008). Currently, only the natriuretic peptides appear to be useful for these purposes. New approaches in bioinformatics, including the use of artificial neural networks, will probably be needed to assist in data analysis and its clinical application.

In September 2010, the University of Texas Health Science Center (San Antonio, TX) received $11.6 million from the National Heart, Lung, and Blood Institute (NHLBI) to conduct a proteomics study to identify protein biomarkers associated with cardiac failure. The study, which will be specifically directed at identifying peptides that may be predictive of individuals who have had a heart attack and who may suffer heart failure later on, is aimed at peptides from the extracellular matrix, whose turnover regulates heart response to injury. Previously, extracellular matrix research has concentrated on identifying cancer biomarkers. In this project, plasma samples will be taken from mice within 1 week after an induced heart attack. The investigators will catalog which extracellular matrix proteins are broken down into peptide fragments after a heart attack, and then research each fragment for biological activity. To do so, the proteomics center is developing new mass spectrometry-based detection methods.

## *Biomarkers for Atherosclerosis*

Atherosclerotic cardiovascular disease is the primary cause of morbidity and mortality, but due to lack of sensitive and specific early biomarkers, the first clinical presentation of more than half of these patients is either myocardial infarction or death. Despite appropriate evidence-based treatments, recurrence and mortality rates remain high even among patients with long established atherosclerotic cardiovascular disease.

### Adipocyte Enhancer-Binding Protein 1

Peroxisome proliferator-activated receptor γ1 (PPARγ1) and liver X receptor α (LXRα) play pivotal roles in macrophage cholesterol homeostasis and inflammation, key biological processes in atherogenesis. Adipocyte enhancer-binding protein 1 (AEBP1) has been identified as a transcriptional repressor that impedes macrophage cholesterol efflux, promoting foam cell formation, via PPARγ1 and LXRα down-regulation (Majdalawieh et al. 2006). Contrary to AEBP1 deficiency, AEBP1 overexpression in macrophages is accompanied by decreased expression of PPARγ1, LXRα, and their target genes ATP-binding cassette A1, ATP-binding cassette G1, apolipoprotein E, and CD36, with concomitant elevation in IL-6, TNFα, monocyte chemoattractant protein 1, and inducible NO synthase levels. AEBP1, represses PPARγ1 and LXRα in vitro. Expectedly, AEBP1-overexpressing transgenic (AEBP1TG) macrophages accumulate considerable amounts of lipids compared

with AEBP1 nontransgenic macrophages, making them precursors for foam cells. These in vitro and ex vivo experimental data strongly suggest that AEBP1 plays critical regulatory roles in macrophage cholesterol homeostasis, foam cell formation, and proinflammation. AEBP1 may be critically implicated in the development of atherosclerosis. As a biomarker for atherosclerosis, AEBP1 may serve as a molecular target toward developing anti-inflammatory, antiatherogenic therapeutic approaches.

### Ghrelin as a Biomarker of Atherosclerosis

Ghrelin, a peptide hormone from stomach, stimulates food intake and decreases fat utilization. Ghrelin binds to growth hormone secretagogue receptor (GHSR). GHSR density has been shown to be upregulated in atherosclerotic lesions. Ghrelin concentrations and carotid artery atherosclerosis are positively associated in males even after adjustment for the commonly recognized risk factors of atherosclerosis (Poykko et al. 2006). Experimental and prospective studies are warranted to elucidate the role of ghrelin in atherosclerosis.

### Imaging Biomarkers of Hypercholesterolemia/Atherosclerosis

Coronary artery calcium (CAC) and carotid intima-media thickness (IMT) are noninvasive measures of atherosclerosis that consensus panels have recommended as possible additions to risk factor assessment for predicting the probability of cardiovascular disease (CVD) occurrence. Atherosclerosis progression from childhood into old age has been followed by measuring intima-media thickness in subjects with familial hypercholesterolemia (FH) and measurement of arterial wall thickness is used as a surrogate marker for atherosclerosis.

A study has investigated the determinants of coronary and carotid subclinical atherosclerosis, aortic stiffness and their relation with inflammatory biomarkers in FH (Martinez et al. 2008). The clinical parameters poorly explained IMT, CAC, and carotid-femoral pulse wave velocity variability in FH. Furthermore, imaging biomarkers and inflammatory biomarkers presented a poor agreement in degree of their severity for CHD prediction. Another study found that CAC score is a better predictor of subsequent CVD events than carotid IMT (Folsom et al. 2008).

An imaging biomarker study by Merck & Co, ACHIEVE (An Assessment of Coronary Health Using an Intima-Media Thickness Endpoint for Vascular Effects), was evaluating MK-0524A (ER niacin/laropiprant) in patients with heterozygous familial hypercholesterolemia. This study was discontinued in 2008 as the patient population was not appropriate for this study.

### Inflammatory Biomarkers of Atherosclerosis

Scientific evidence and findings from both clinical and population studies indicate that chronic inflammation contributes to the development and progression of cardiovascular diseases. Inflammation has been implicated in all stages of atherosclerosis and elevated serum inflammatory biomarkers have been used to assess

cardiovascular risk and response to therapy. However, most of the current known inflammatory biomarkers are not useful in screening for atherosclerotic disease. C-reactive protein, sedimentation rate, and fibrinogen are not derived from the vasculature and may signal inflammation in any organ. It is also possible that due to heterogeneity among the population at risk, a single marker cannot provide sufficient information for accurate prediction of disease. Therefore, there is a need for identification of inflammatory markers that are more specific to vascular disease and can be used for highly sensitive and specific assays capable of detecting and quantifying atherosclerotic cardiovascular disease. Measurement of multiple circulating disease-related inflammatory factors may be more informative, enabling the early identification of vascular wall disease activity.

### Lipid-Modified Proteins as Biomarkers of Atherosclerosis

The biological function of lipid-modified proteins depends on the identity of the attached lipid. At least five different lipid modifications of cysteines, glycines, and other residues on the COOH- and NH(2)-terminal domains have been described. Available evidence suggests that lipid-modified proteins are directly involved in different steps of the development of lesions of atherosclerosis, from leukocyte recruitment to plaque rupture, and their expression or lipid modification are likely altered during atherogenesis (Ferri et al. 2005). Several lipid-modified proteins can be used as biomarkers for atherosclerosis.

### Lp-PLA2 as Biomarker of Atherosclerotic Heart Disease

Lipoprotein-associated phospholipase A2 (Lp-PLA2) is a member of the phospholipase A2 superfamily, a family of enzymes that hydrolyze phospholipids. Circulating Lp-PLA2 is a marker of inflammation that plays a critical role in atherogenesis; its inhibition may have antiatherogenic effects. Epidemiological data have consistently demonstrated the association of increased levels of Lp-PLA2 with increased risk of coronary heart disease (Sudhir 2005). Polymorphisms of the Lp-PLA2 gene have been reported, with varying significance, in Japanese and Caucasian populations. Overall, epidemiological studies suggest that measurement of Lp-PLA2 in plasma may be a useful biomarker for identifying individuals at high risk for cardiac events. PLAC test (diaDexus Inc) for detection of Lp-PLA2 in plasma is effective as a predictive biomarker of risk for cardiovascular disease and approved by the FDA.

### Nitric Oxide Impairment and Atherosclerosis

The role of nitric oxide (NO) in the cardiovascular system and in atherosclerosis has been described in detail in a special report (Jain 2010). Many studies have shown that NO is a major antioxidant that serves to block oxygen-free radicals, which, among other things, create oxidized low-density lipoprotein (LDL) cholesterol

that damages the endothelium and leads to atherosclerosis. The measurement of NO bioavailability is of great clinical interest in the assessment of vascular health. However, NO is rapidly oxidized to form nitrite and nitrate and thus its direct detection in biological systems is difficult.

### Oxygen Free Radicals as Biomarkers of Atherosclerosis

Excessive production and/or inadequate removal of ROS, especially superoxide anion, have been implicated in the pathogenesis of many cardiovascular diseases, including atherosclerosis, hypertension, diabetes, and in endothelial dysfunction by decreasing NO bioactivity. Vascular aging may be related to oxidative stress. Age-related morphologic changes in large resistance vessels include an intima-media thickening, increased deposition of matrix substances, thus ultimately leading to a reduced compliance. Vascular aging is mainly characterized by an impaired endothelium-dependent vasorelaxation. The expression of endothelial nitric oxide synthase (eNOS), producing vasodilatatory NO, is markedly upregulated with increasing age. However, vasorelaxation is impaired, as the production of ROS such as superoxide ($O_2$-), concomitantly increases.

### Proteomic Profiles of Serum Inflammatory Markers of Atherosclerosis

Protein microarray-based measurements of abundant circulating proteins have been carried out to identify biomarkers of atherosclerotic disease. Using a longitudinal experimental design with apoE-deficient mice and control serum protein expression of 30 inflammatory markers measured by protein microarray, a subset of proteins was identified to predict severity of atherosclerotic disease with high level of accuracy (Tabibiazar et al. 2006). The top serum protein biomarkers in this study cover a wide range of atherosclerotic biological process including macrophages chemoattraction, T-cell chemokine activity, innate immunity, vascular calcification, angiogenesis, and high fat-induced inflammation. The signature pattern derived from simultaneous measurement of these markers, which represent diverse atherosclerosis-related biological processes, will likely add to the specificity needed for diagnosis of atherosclerotic disease. The time specific vascular expression of these markers was verified by showing their gene expression in the mouse aorta correlated closely to the temporal pattern of serum protein levels. These data suggest that quantification of multiple disease-related inflammatory proteins will provide a more sensitive and specific method for assessing atherosclerotic disease activity in humans, and identify candidate biomarkers for such studies. Because the vascular tissue is not readily accessible, identification of protein markers in the serum can have practical implications in developing diagnostic tools for diagnosis of coronary artery disease in humans. A detailed microarray-based picture of the transcriptional landscape in the diseased tissue would be useful for assessing upstream components in the pathways that lead to inflammatory mediator expression, which is the first step in developing highly targeted therapeutics. Serum biomarker assays can then be used to assess the effects of such therapeutics.

## *Biomarkers of Risk Factors for Coronary Heart Disease*

### Antibody to Oxidized-LDL

Following rupture of the plaque oxidised-LDL (Ox-LDL), stored in the wall of the plaque, sets off an immunological reaction in the blood stream and antibodies are produced, which are directed against the exposed Ox-LDL molecules. These antibodies can be measured in the laboratory as biomarkers. One particular form of antibody is known to be particularly associated with the risk of a heart attack. EG010 (Ark Therapeutics) has been specifically developed as an IVD and optimized to detect this antibody. Initial trials data indicate that it predicted 81% of patients with an acute coronary syndrome, comparing very favorably with CRP testing, which only predicted 29%. Ark has completed the necessary clinical validation work to achieve CE marking status in Europe and will address the US position once it has obtained CE marking in Europe.

### Apolipoproteins as Risk Factors for Coronary Heart Disease

Apolipoproteins AI and B are structural components of lipoprotein particles, and also determinants of the metabolic fate of the encapsulated lipid, cholesterol, and triglyceride. Development of accurate assays for these apolipoproteins has opened the way for their use as predictors of coronary heart disease risk. Interpretation of AI and apo B levels is best undertaken with background knowledge of the metabolic status of an individual, especially the lipolytic capacity as reflected in the triglyceride concentration. Those with raised triglyceride, in general, not only have an elevated apo B/apo AI ratio, but also apo B-containing lipoproteins with a prolonged residence time and hence ample opportunity for modification and damage (Marcovina and Packard 2006). Assessment of apolipoprotein levels is an aid to risk prediction and can be useful in tailoring treatment. An inverse relationship between the concentration of high-density lipoprotein (HDL) cholesterol and the risk of developing cardiovascular is well established. There are several documented functions of HDLs that may contribute to a protective role of these lipoproteins. These include the ability of HDLs to promote the efflux of cholesterol from macrophages and foam cells in the artery wall and to anti-inflammatory/antioxidant properties of these lipoproteins. The fact that the main apolipoprotein of HDLs, apoA-I, plays a prominent role in each of these functions adds support to the view that apoA-I should be measured as a component of the assessment of cardiovascular risk in humans (Barter and Rye 2006).

Results from recent epidemiological studies and statin trials suggest that apolipoprotein B-100 (apoB), with or without apoA-I, is superior to LDL cholesterol in predicting coronary events (Chan and Watts 2006). Measurements of apolipoproteins are internationally standardized, automated, cost-effective and more convenient and precise than those for LDL cholesterol. ApoB may also be preferable to the measurement of non-HDL cholesterol. Measurement of apolipoproteins (apoB and possibly apoA-I) should be routinely added to the routine lipid profile (cholesterol,

triglycerides, and high-density lipoprotein cholesterol) to assess the atherogenic potential of lipid disorders. This is particularly relevant to dyslipidemias characterized by an elevation in plasma triglycerides. Apolipoproteins, especially apoB, could also replace the standard "lipid profile" as a target for therapy in at-risk patients.

### CRP as Biomarker of Risk for Coronary Heart Disease

C-reactive protein (CRP) levels have been considered as a biomarker of coronary artery disease but this was somewhat controversial until recently. In an epidemiologic study, among those whose CRP levels were among the top one-fourth of participants, there was a 66% increased adjusted risk of developing coronary artery disease compared to the risk experienced by those whose levels were in the lowest fourth (Boekholdt et al. 2006). When fatal coronary artery disease risk was separately examined, it was found to be nearly three times greater among participants whose levels were in the top fourth of CRP levels than those whose levels were lowest, making CRP a more predictive risk factor than even smoking and diabetes among this group. The results indicate that the predictive value of CRP plasma levels may be stronger for mortality than for total coronary artery disease incidence.

High sensitivity CRP (hsCRP) is an alpha globulin that is synthesized in the liver and present as a trace constituent in the blood. Levels of hsCRP rise during general, nonspecific response to a wide variety of diseases, therefore elevated hsCRP is not specific to any particular disease. Despite this limitation, hsCRP is a useful indicator of the inflammatory processes that usually accompany cardiovascular disease and, when monitored in conjunction with the other cardiac biomarkers, can aid in diagnosis. Prospective studies have shown hs-CRP to be a predictor of increased cardiovascular risk in both men and women and that it may be a better predictor of the risk for heart attacks than cholesterol. Evidence suggests patients with elevated hs-CRP and normal cholesterol levels are at greater risk than those with normal hs-CRP and high cholesterol levels. Achieved levels of hsCRP at 30 days and 4 months after acute coronary syndrome are independently associated with long-term survival (Morrow et al. 2006). Patients treated with more aggressive statin therapy are more likely to achieve lower levels of hsCRP. In a prospective study of initially healthy women, baseline levels of fibrinogen measured with a high-quality immunoassay provided additive value to hs-CRP and traditional risk factors in predicting incident cardiovascular disease (Mora et al. 2006a).

### Impairment of EPCs by Oxidative Stress as a Biomarker of Disease

Circulating endothelial progenitor cells (EPCs) in adult human peripheral blood have been extensively studied as biomarkers to assess the risk of cardiovascular disease in human subjects and as a potential cell therapeutic for vascular regeneration. EPCs are exposed to oxidative stress during vascular injury as residents of blood vessel walls or as circulating cells homing to sites of neovascularization. Given the links between oxidative injury, endothelial cell dysfunction, and vascular

disease, recent investigation has focused on the responses of EPCs to oxidant stress and the molecular mechanisms that control redox regulation in these specialized cells. Various cell and flow cytometric techniques have been used to define and isolate EPCs from circulating blood and the current human and mouse genetic data, which offer insights into redox control in EPC biology and angiogenesis (Case et al. 2008). EPC responses to oxidant stress may be a critical determinant in maintaining the integrity and function of the cardiovascular system, and perturbations of redox control in EPCs may lead to human diseases. High cholesterol causes increased oxidative stress, impairing the function of EPCs. In addition to being implicated in cardiovascular diseases, oxidative stress is also a factor in diabetes. A comprehensive understanding of how oxidative stress, the biochemical modification of cells, impairs EPC function may lead to antioxidant therapy to prevent cardiovascular disease. These strategies will need to be applied early in the disease when preventing oxidative damage is a possibility because once the damage has occurred it may not be reversible. Eventually it should be possible to do a simple blood test to measure EPCs to determine the risk for disease.

## *Biomarkers for Pulmonary Arterial Hypertension*

Pulmonary arterial hypertension (PAH) is defined as a group of diseases characterized by a progressive increase in pulmonary vascular resistance leading to right ventricular failure and premature death. The cause of primary pulmonary hypertension is not definitely known, but HSV-8 infection in immunocompromised individuals has been linked to it. There are several causes of secondary pulmonary hypertension, but it is usually due to an underlying heart condition, lung disease, or pulmonary embolism. The diagnostic procedures include ECG, chest X-ray, Doppler echocardiography, pulmonary function tests, arterial blood gas analysis, computed tomography of the lungs, and pulmonary angiography. Biomarkers have been proposed to reliably monitor the clinical course.

Plasma BNP is elevated in cardiac right ventricular (RV) dysfunction and plays a key role in protecting the body from volume overload by maintaining renal function and sodium balance. Studies in patients with PAH have demonstrated that plasma BNP levels are raised proportionally to the extent of RV dysfunction (Hargett and Tapson 2005). There is growing evidence that BNP may be a potential biomarker for PAH in screening for occult disease, diagnostic evaluation, prognosis, and estimating a response to therapy. Additionally, augmentation of the natriuretic peptide system through exogenous administration of BNP or by preventing its degradation may be a promising option for the management of decompensated RV failure. Because plasma BNP levels rise in a variety of cardiopulmonary conditions and are affected by several physiological factors, BNP interpretation must not occur in isolation but rather within the context of clinical diagnosis.

University of Colorado scientists have devised effective and precise algorithms for subtyping PAH pathologies and prognosis by use of a noninvasive blood sample

with a panel of indicative biomarkers. Microarray expression was performed, and the expression profiles were analyzed for consistent and predictive differences in gene expression. They identified a signature set of 106 genes that discriminated with high certainty between patients with PAH and normal individuals (Bull et al. 2004). The results of the microarray analysis were retrospectively and prospectively confirmed by quantitative PCR for 2 of the 106 genes. Supervised clustering analysis generated a list of differentially expressed genes between patients with idiopathic and secondary causes of PAH. These findings may have important implications toward diagnosis, screening, and pathogenesis of this disease. Ultimately, the diagnostic platform would ideally be based upon a high throughput, low cost system detecting the known and validated changes in gene expression of biomarkers associated with the disease of interest. This format would be precise, accurate, scalable, portable, and widely feasible. Alternatively, the technology could be rolled into a more extensive panel of similar diagnostics, that is, a broad pulmonary biomarker array on a probe array or Affymetrix chip, for more extensive applications in clinical diagnosis, drug development, or biomedical research. The technology has potential applications in clinical drug development, diagnostics (from risk-factor determination to disease staging), and personalized medicine. This technology is available for licensing.

### Biomarkers of Abdominal Aortic Aneurysm

Abdominal aortic aneurysm (AAA) is the term used for significant ballooning of the aorta, the largest artery in the body, which may lead to rupture. AAA is the cause of more than 15,000 deaths each year in the USA placing it as the 15th leading cause of death. Patients considered high-risk for AAA include older men with a history of cigarette smoking, high blood pressure and cholesterol, and with a family history of the disease. AAAs are difficult to diagnose because of their location deep within the abdomen, and are usually only detected by chance, when ultrasound, CT, or MRI imaging techniques are performed for other reasons. Because these imaging techniques are relatively expensive, they are not regularly performed to search for aneurysms, even in high-risk patients. AAAs may also be detected when they become very large and rupture or are in immediate danger of rupturing, in which case emergency surgery is required and is often unsuccessful in saving the patient.

Elective surgical treatment is recommended on the basis of an individual's risk of rupture, which is predicted by AAA diameter. However, the natural history of AAA differs between patients and a reliable and individual predictor of AAA progression (growth and expansion rates) has not been established. Several circulating biomarkers are candidates for an AAA diagnostic tool. However, they have yet to meet the triad of biomarker criteria: biological plausibility, correlation with AAA progression, and prediction of treatment effect on disease outcome. If a cost-effective screening tool could detect aneurysms early, it would significantly reduce aneurysm-related mortality.

Circulating levels of biomarkers of extracellular matrix degeneration, such as elastin peptides, aminoterminal propeptide of type III procollagen, elastase-alpha1-antitrypsin complexes, matrix metalloproteinase 9, cystatin C, plasmin–antiplasmin complexes, and tissue plasminogen activator, have been correlated with AAA progression and have biological plausibility (Hellenthal et al. 2009a). Role of direct and indirect biomarkers of inflammation including various cytokines, C-reactive protein, activators of tissue plasminogen activator and urokinase plasminogen activator, and osteopontin has also been examined in progression AAA (Hellenthal et al. 2009b).

Seeing a need for a more widely available screening tool, scientists at University of Virginia have conducted research to develop an easy, clinical diagnostic test to screen patients for abdominal aortic aneurysm before their conditions become dire. They identified 119 protein-based biomarkers in the plasma that could help alert physicians to the presence of an AAA with a simple blood test. Seeking to further develop and ultimately commercialize their technology, they have licensed this technology to Ortho Clinical Diagnostics Inc that would allow testing of the aneurysm biomarkers on a much greater scale.

## *Genetic Biomarkers for Cardiovascular Disease*

### Genetic Biomarkers of Atherosclerosis

Finding genes that influence systemic levels of inflammatory biomarkers may provide insights into genetic determinants of atherosclerosis. Variance-component linkage analyses of blood levels of four biomarkers of vascular inflammation – CRP, IL-6, MCP-1, sICAM-1 – in extended families from the Framingham Heart Study showed that multiple genes on chromosome 1 may influence inflammatory biomarker levels and may have a potential role in the development of atherosclerosis (Dupuis et al. 2005).

Researchers at the Harvard Medical School (Boston, MA) have identified several genes that are involved in the development of endothelium of blood vessels; dysfunction of endothelial lining is a primary cause of cardiovascular disease. Lesions of atherosclerosis are isolated to atheroprotective and atheroprone regions. The blood flow is laminar in atheroprotective region and lesions do not form whereas the flow is turbulent in atheroprone regions leading to atherosclerosis. They studied the two regions by genome-wide transcriptional profiling using gene expression arrays from Life Technologies Corp and analyzed the differences in expression of transcription factors to identify potential biomarkers. They found that the transcription factor Kruppel-like factor 2 (KLF2) is selectively induced in endothelial cells exposed to a biomechanical stimulus characteristic of atheroprotected regions of the human carotid and this flow-mediated increase in expression occurs via a MEK5/ERK5/MEF2 signaling pathway (Parmar et al. 2006). Overexpression and silencing of KLF2 in the context of flow, combined with findings from genome-wide analyses of gene expression, demonstrate that the induction

of KLF2 results in the orchestrated regulation of endothelial transcriptional programs controlling inflammation, thrombosis/hemostasis, vascular tone, and blood vessel development. These data also indicate that KLF2 expression globally modulates IL-1beta-mediated endothelial activation. KLF2 therefore serves as a mechano-activated transcription factor important in the integration of multiple endothelial functions associated with regions of the arterial vasculature that are relatively resistant to atherogenesis.

### IL-1 Gene Polymorphism as Biomarker of Cardiovascular Disease

IL-1 is one of the body's most potent natural mechanisms for controlling the inflammatory response to injury. Inflammation is a crucial factor in the process that leads to heart disease and subsequent heart attacks. In 2000, Interleukin Genetics Inc was awarded a notice of allowance from the US Patent and Trademark Office for the discovery of the effect of IL-1 gene variations in increasing the risk of heart disease. Studies conducted by Interleukin Genetics have demonstrated that certain variations in the IL-1 gene can amplify inflammation in the arteries, and make an individual two to four times more likely to develop clinically significant heart disease prior to age 60. It has been implicated in essential hypertension and heart failure. The Gensona™ Heart Health Genetic Test (Interleukin Genetics) is the first and only IL1 gene test to identify an individual's predisposition for overexpression of inflammation and increased risk for cardiovascular disease.

### Polymorphisms of the eNOS Gene and Angina Pectoris

Angina pectoris is chest pain due to transient myocardial ischemia. It may be a stable chronic manifestation with angina occurring on exertion or unstable form of angina with pain at rest, which may be due to coronary artery disease. Coronary artery spasm plays an important role in the pathogenesis of vasospastic angina, and contributes to the development of several acute coronary syndromes. Endothelial nitric oxide synthase (eNOS) catalyzes the synthesis of NO, which regulates vascular tone, and may be related to coronary vasospasm. Coronary spasm may be related to particular polymorphisms of the eNOS gene. Two genotypes in intron 4 of eNOS (NOS4a), a/a or a/b are significant predictors of coronary spasm (Kaneda et al. 2006). In patients with NOS4a, both the induced and spontaneous contractions are augmented, indicating that NOS4a could be a good biomarker for coronary artery spasm.

### Polymorphisms in the Apolipoprotein E Gene

Apolipoprotein E (APOE) acts as a ligand for the LDL receptor and has an important role in clearing cholesterol-rich lipoproteins from plasma. The $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ alleles of the apolipoprotein APOE gene encode three isoforms, apoE2, E3, and E4, respectively. The apoE isoforms circulate in different plasma concentrations, but

plasma concentrations of the same isoform also differ between individuals. This genetic variation is associated with different plasma lipoprotein levels, different response to diet and lipid-lowering therapy, and a variable risk for cardiovascular disease. The E4 allele of the apolipoprotein E gene occurs in about 30% of the general population (E3 being the most common allele). Heterozygotes possessing alleles E3/E4 have cholesterol concentrations on average 10% higher than those of E3/E3 homozygotes because of the differential allelic effects on lipoprotein particle turnover. This difference extends to coronary events, although the risk associated with the E4 allele seems greater than is indicated from its effect on cholesterol levels. A third version of the gene, the E2 allele, occurs in its homozygous form. When combined with a second risk factor such as diabetes or hypothyroidism, the E2/E2 genotype results in type III dysbetalipoproteinemia, which is associated with a high risk of coronary and peripheral vascular disease and occurs in 0.01–0.02% of the population.

In old age, high plasma apoE levels precede an increase of circulating CRP and strongly associates with cardiovascular mortality, independent of APOE genotype and plasma lipids (Mooijaart et al. 2006). ApoE polymorphism is also a risk factor for Alzheimer disease.

### Mutations in the Low Density Lipoprotein Receptor Gene

Familial hypercholesterolemia is an autosomal dominant disease defined at the molecular level mainly by the presence of mutations in the LDL receptor gene. More than 600 mutations in the LDLR gene have been identified in patients with this disorder. One in 500 people is heterozygous for at least one such mutation, whereas only one in a million is homozygous at a single locus. Those who are heterozygous produce half the normal number of LDL receptors, leading to an increase in plasma LDL levels by a factor of 2 or 3, whereas LDL levels in those who are homozygous are six to ten times normal levels. Affected individuals typically have cholesterol concentrations twice the population average for their age and sex. LDL is the major cholesterol-carrying lipoprotein in plasma and is the causal agent in many forms of coronary heart disease. Four monogenic diseases raise plasma levels of LDL by impairing the activity of hepatic LDL receptors, which normally clear LDL from the plasma.

There is an interest in developing diagnostic systems for LDL genotyping, which will enable identification of responders to statin therapy and those at increased risk of adverse drug reactions or patients.

### Mutations Within Several Genes That Code for Ion Channel

Inherited cardiac rhythm disorders are a group of genetically determined diseases due to mutations in genes coding for various cardiac ion channels. The most common cardiac ion channel disease is the long QT syndrome. Cardiac ion channel disorders may lead to sudden cardiac death. Prophylactic and life-saving therapies are available for many of these disorders. Therapy and risk stratification depend

on the clinical presentation, the ECG pattern, and which gene is mutated. Genetic testing offers the opportunity to exclude individual family members as mutation carriers.

Following earlier studies of the involvement of some cardiac ion currents in adverse drug interactions, recent reports have identified not only the ion channel subunits involved but also a range of mutations and SNPs in ion channel genes that predispose to both drug-induced and familial cardiac arrhythmia.

### Polymorphism in the Angiotensinogen Gene

The renin-angiotensin system plays a central role in health and disease but the determinants of renin-angiotensin system activity have not been fully elucidated. A common variant of the angiotensinogen gene (T235) predicts elevated levels of circulating angiotensinogen, and polymorphisms of this gene have been linked to physiologic responses and to the risk of cardiovascular disease There is an association between angiotensinogen M235T polymorphism and coronary artery disease severity independently of other cardiovascular risk factors (Lanz et al. 2005). T235 is also a genetic marker for early carotid atherosclerosis in a hypertensive population, which has been shown to regression under antihypertensive treatment.

### Kallikrein Gene Mutations in Cardiovascular Disease

All the components of the kallikrein-kinin system are located in the cardiac muscle, and its deficiency may lead to cardiac dysfunction. Muations of the kallikrein gene are associated with several diseases. In recent years, numerous observations obtained from clinical and experimental models of diabetes, hypertension, cardiac failure, ischemia, myocardial infarction, and left ventricular hypertrophy have suggested that the reduced activity of the local kallikrein-kinin system may be instrumental for the induction of cardiovascular-related diseases. The cardioprotective property of the angiotensin-converting enzyme inhibitors is primarily mediated via kinin-releasing pathway, which may cause regression of the left ventricular hypertrophy in hypertensive situations. The ability of kallikrein gene delivery to produce a wide spectrum of beneficial effects makes it an excellent candidate in treating cardiovascular diseases (Sharma 2006).

### Kallikrein Gene and Essential Hypertension

Ten alleles with length and nucleotide sequence variations were identified in the regulatory region of human tissue kallikrein gene. There are polymorphisms in the regulatory region of human tissue kallikrein gene in the Chinese Han people. Differences in both allele frequencies and genotype frequencies between these two groups have provided evidence toward the association of hypertension with the polymorphisms in this studied site (Hua et al. 2005).

US Patent No. 5,948 from Medical University of South Carolina (Charleston, SC) describes a biomarker for identifying a human subject as having an increased

or decreased risk of developing essential hypertension. This is carried out by determining the presence in the subject of an allele in the promoter region of the tissue kallikrein gene, which is correlated with an increased or decreased risk of developing essential hypertension. US Patent No. 6,747,140 describes the susceptibility to develop hypertension associated with a mutation in the kallikrein gene.

### Gene Mutations in Pulmonary Arterial Hypertension

Mutations in bone morphogenetic protein receptor type-2 (BMPR2) are found in about 50% of patients with familial PAH (Newman et al. 2004). Because familial PAH is highly linked to chromosome 2q33, it is likely that the remaining 50% of family cases without exonic mutations have either intronic BMPR2 abnormalities or alterations in the promoter or regulatory genes. Also, only about 10% of patients with "sporadic" idiopathic PAH have identifiable BMPR2 mutations. Mutations in BMPR2 confer a 15–20% chance of developing PAH in a carrier's lifetime. Advances in genetic testing, presymptomatic screening, and biomarkers should permit early detection of disease in those at risk of PAH and allow trials of preventive therapy in carriers.

### Genetic Biomarkers of Early Onset Myocardial Infarction

Two novel genetic biomarkers, SNPs, are associated with an increased risk for MI: VAMP8, which is involved in platelet degranulation, and HNRPUL1, which encodes a ribonuclear protein (Shiffman et al. 2006). The retrospective research study was performed on samples from over 2,000 individuals in three case-control studies to compare patterns of genetic variation in people with a history of early-onset MI to those with no history of MI. The results were significant in all three studies. Large-scale studies like this one, with well-characterized samples from carefully selected patients allow the identification of genetic markers for risk of early-onset MI, which could potentially be incorporated into individual risk assessment protocol. The identification of these variants could improve understanding of disease mechanisms and suggest novel drug targets.

### Gene Variant as a Risk Factor for Sudden Cardiac Death

Extremes of the electrocardiographic QT interval, a measure of cardiac repolarization, are associated with increased cardiovascular mortality. A gene called NOS1AP (CAPON) that may predispose some people to abnormal heart rhythms, which leads to sudden cardiac death was identified through a genome-wide association study (Arking et al. 2006). Statistically significant findings were validated in two independent samples of 2,646 subjects from Germany and 1,805 subjects from the US Framingham Heart Study. NOS1AP, a regulator of neuronal nitric oxide synthase (nNOS), modulates cardiac repolarization. The gene, not previously discovered by traditional gene-hunting approaches, appears to influence significantly QT interval length as risk factor for sudden cardiac death. QT interval can be measured

noninvasively with an EKG, and each person's QT interval, in the absence of a major cardiovascular event, is stable over time, making it a reliable measure. Approximately, 60% of subjects of European ancestry carry at least one minor allele of the NOS1AP genetic variant, which explains up to 1.5% of QT interval variation.

Instead of focusing on so-called candidate genes with known functions that are highly suspect in heart beat rhythm, the researchers first focused on people who have extremely long or short QT intervals. They used subjects from two population-based studies, about 1,800 American adults of European ancestry from the Framingham Heart Study of Framingham, Massachusetts, and about 6,700 German adults from the KORA-gen study of Augsburg, Germany. They looked at SNPs that track with having a long or short QT interval. Only one particular SNP correlated with QT interval. That SNP was found near the NOS1AP gene, which has been studied for its function in nerve cells and was not previously suspected to play a role in heart function.

Identifying those at high risk for sudden cardiac death before fatalities occur has been challenging, both at the clinical and at the genetic level. In more than one third of all cases, sudden cardiac death is the first hint of heart disease. It is widely believed that many factors, genetic and environmental, contribute to irregular heartbeat and other conditions that may lead to sudden cardiac death. Now that variants of the NOS1AP gene have been correlated with QT interval length, the next project would be to figure out exactly how the DNA sequence variations alter the function of the gene, and how changes in gene function affects heart rhythm. Being able to identify predisposed individuals can save their lives by prescribing beta-blockers and other drugs that regulate heart rhythm, and even by implanting automatic defibrillators in those with the highest risk.

## *Multiple Biomarkers for Prediction of Death from Cardiovascular Disease*

Multiple biomarkers from different disease pathways may be involved in fatal outcome from cardiovascular disease. Uppsala Longitudinal Study of Adult Men (ULSAM), a community-based cohort of elderly men, has shown that a combination of biomarkers that reflect myocardial cell damage, left ventricular dysfunction, renal failure, and inflammation (troponin I, N-terminal pro-brain natriuretic peptide, cystatin C, and C-reactive protein, respectively) improved the risk stratification of a person beyond an assessment that was based on the established risk factors for cardiovascular disease (Zethelius et al. 2008).

In addition to biomarkers, various well-validated scoring systems based on clinical characteristics are available to help clinicians predict mortality risk, such as the Thrombolysis in Myocardial Infarction score and Global Registry of Acute Coronary Events score. A multimarker approach incorporating biomarkers and clinical scores will increase the prognostic accuracy.

## Role of Biomarkers in the Management of Cardiovascular Disease

### *Role of Biomarkers in the Diagnosis/Prognosis of Myocardial Infarction*

According to the World Health Organization, the diagnosis of myocardial infarction requires that two out of the following three criteria be met:

1. Clinical history of ischemic type chest pain for at least 20 min
2. Changes in serial ECG tracings
3. Rise and fall of serum cardiac enzymes (biomarkers)

BNP/NT-proBNP and CRP are mentioned in the current guidelines of the European Society of Cardiology as appropriate biomarkers for risk stratification of myocardial infarction, whereas clinical relevance of other novel biomarkers remains uncertain and necessitates further studies (Weber and Hamm 2008).

According to a 2009 Henry Ford Hospital (Detroit, MI) study, many patients with myocardial infarction have high levels of cardiac biomarkers in the blood for several months after leaving the hospital, with more shortness of breath and chest pain. The study examined a subset of patients in a 4,500-patient heart attack registry from 24 US hospitals and found that 6 months later 9% percent had elevated levels of the TnT and 33% had elevated level of the BNP/NT. These elevated biomarkers were definitely associated with a reduced quality of life for patients and worse outcomes. This data raises two important issues; (1) whether the biomarkers are a sign of ongoing problems or a reflection of the past myocardial infarction, and (2) whether closer monitoring of patients post-myocarial infarction can help target the treatment to those who need it most.

### *Role of Biomarkers in the Prevention of Cardiovascular Disease*

Preventive treatment for those most at risk of heart disease rather than those with the highest blood pressure or cholesterol values may be a more efficacious strategy for disease management. This depends on accurate biomarker-based risk assessment tools. An evidence-based model of heart disease risk was developed using the Framingham model with an additional five risk factors, including three of the newer blood biomarkers (Root and Smith 2005). This was applied to the adult population of the 3rd National Health and Nutrition Examination Survey cohort. Additionally, the selection criteria for therapeutic intervention from the Adult Treatment Panel III guidelines (for hyperlipidemia) and the 7th Report of the Joint National Committee (for hypertension) were applied to the same subjects. Of this cohort,

54% qualified for at least one of these medications while 18% qualified for both. Using this 18% cutoff, the 18% of the subjects with the highest calculated heart disease risk were also identified using the developed risk model. Applying both drugs to the high-risk group (one third the size of the guidelines group) achieved the same reduction in population risk (about one fourth) as applying the drugs to the guideline groups and required only half as many prescriptions. Intermediate results were found when an intervention group was identified by a combination of both high risk and high levels of risk factors. In this simulation, identifying patients by heart disease risk level resulted in substantially fewer people being treated with fewer drugs and achieving a similar reduction in disease risk.

The benefit of a biomarker-based risk model is that the newest risk factor information can be incorporated into the model and a more accurate assessment of risk made. The cost of the biomarkers is an added cost not encountered in the guidelines-based approach. However, this increase is more than compensated for by the 44% reduction in cardiovascular disease events in the high-risk group.

A study was conducted to determine the association of physical activity and body mass index (BMI) with novel and traditional cardiovascular biomarkers in women (Mora et al. 2006b). Biomarkers used in the study included hsCRP, fibrinogen, soluble ICAM-1, homocysteine, LDL and HDL cholesterol, total cholesterol, apolipoprotein A-1 and B100, lipoprotein-a, and creatinine. Lower levels of physical activity and higher levels of BMI were independently associated with adverse levels of nearly all lipid and inflammatory biomarkers. High BMI showed stronger associations with these biomarkers than physical inactivity. However, within BMI categories, physical activity was generally associated with more favorable cardiovascular biomarker levels than inactivity.

## *Molecular Signature Analysis in Management of Cardiovascular Diseases*

In cardiovascular disorders, microarray studies have largely focused on gene discovery, identifying differentially expressed genes characteristic of diverse disease states, through which novel genetic pathways and potential therapeutic targets may be elucidated. However, gene expression profiling may also be used to identify a pattern of genes (a molecular signature) that serves as a biomarker for clinically relevant parameters (Kittleson and Hare 2005). Molecular signature analysis (MSA) accurately predicts the etiologic basis of heart failure and cardiac transplant rejection. These early studies provide valuable proof of concept for future work using MSA. The ultimate potential application of transcriptome-based MSA is individualization of the management of patients with structural heart disease, arrhythmias, and heart failure. A patient with a newly diagnosed cardiac disease could, through molecular signature analysis, be offered an accurate assessment of prognosis and how individualized medical therapy could affect the outcome.

## *C-Reactive Protein as Biomarker of Response to Statin Therapy*

CRP levels are low in healthy young people – usually less than 1 mg/L of blood – but they rise with age, obesity, diabetes, smoking, and a sedentary life. If people lose weight, stop smoking, exercise, or take oral diabetes drugs, their CRP levels fall. But a third of the population has levels greater than 3 mg/L, and levels that high have been associated with heart disease risk. Questions remain as to the CRP's normal role in the body. It was discovered about 70 years ago by scientists who were trying to understand why some streptococci caused disease and others did not. It is so named because it was found in the third band, called Band C, in a gel used to separate proteins. Then, about half a century ago, physicians noticed that CRP flooded the patient's blood after a heart attack, and for several years, the protein was used to help diagnose heart attacks.

Two studies have shown that reducing the levels of CRP, which plays a role in heart disease, may be as powerful a tool in slowing heart disease and preventing heart attacks and cardiac-related death as lowering cholesterol (Ridker et al. 2005; Nissen et al. 2005). The participants were patients with severe heart disease who were taking high doses of statin drugs, which reduce both cholesterol and CRP. In both studies, CRP independently predicted heart disease progression. Lower CRP levels were linked to a slower progression of atherosclerosis and fewer heart attacks and deaths. And this effect was independent of the effect of lowering cholesterol. This is hard clinical evidence that reducing CRP is at least as important as lowering cholesterol. These findings indicate that physicians should monitor CRP levels in patients with severe heart disease and do whatever it takes, including giving high doses of the most powerful statins, to get levels below 2 mg/L blood.

However, more work is needed to prove that CRP directly causes heart disease. The studies involved only people with severe heart disease; therefore, it remained unknown whether healthy people would benefit from reducing their CRP levels. It is also possible that CRP is a biomarker for some other abnormality that is corrected by statin drugs to reduce heart disease risk. Even before these studies, evidence had been accumulating that CRP and heart disease were somehow linked. There were hypotheses to explain why the protein could cause plaque to develop in coronary arteries, lead plaque to burst open or bring on the formation of blood clots, which block arteries and cause heart attacks. Some pharmaceutical companies have started programs to develop drugs that make a specific target of CRP and prevent its synthesis. That CRP levels drop with exercise and weight loss has led some to argue that the protein is a biomarker of heart disease risk, not a cause. CRP is made in the liver and also in the walls of coronary arteries and possibly elsewhere in the body. Its levels, which can be measured with a simple blood test, often rise and remain high in patients who have chronic inflammation from conditions like rheumatoid arthritis, for example, or periodontal disease. Patients with chronic inflammation also have an increased risk of heart disease. The next step is to see if reducing CRP levels can prevent heart attacks in healthy people. A new study will enroll 15,000 people with normal cholesterol levels but higher-than-average levels of CRP, >2 mg/L blood. The participants will be randomly assigned to take either a statin or a placebo.

## *Role of Circulating Biomarkers and Mediators of Cardiovascular Dysfunction*

Both circulating biomarkers and mediators of cardiovascular dysfunction play a role in acute illness. Some of these circulating biomarkers reflect mediator action on the peripheral vasculature, such as endothelium-derived endothelin and nitrite/nitrate, the stable end products of NO. Other biomarkers mainly reflect actions on the heart, such as the natriuretic peptide family, released from the heart upon dilatation, serving as a marker of congestive heart failure. Some factors may be both markers as well as mediators of cardiovascular dysfunction of the acutely ill and bear prognostic significance. Assessing circulating levels may help refine clinical judgment of the cardiovascular derangements encountered at the bedside, together with clinical signs and hemodynamic variables (Beishuizen et al. 2005). For instance, assessing natriuretic peptides in patients with pulmonary edema of unclear origin may help to diagnose congestive heart failure and cardiogenic pulmonary edema, when the pulmonary capillary wedge pressure is not measured or inconclusive. Future aligning of hemodynamic abnormalities with patterns of circulating cardiovascular markers/mediators may help to stratify patients for inclusion in studies to assess the causes, response to therapy, and prognosis of cardiovascular derangements in the acutely ill patients.

## *Use of Protein Biomarkers for Monitoring Acute Coronary Syndromes*

Although it is easy to diagnose myocardial infarction, no accurate noninvasive efficient method of detecting acute coronary disease in an emergency or outpatient setting in patients with minimal or nonspecific symptoms is available as yet. Many of these patients are discharged without further investigations. They are considered to be low risk as only 2–5% of patients who develop myocardial infarction later on, initially present in this manner and are discharged home. However, more than 5% of patients who present with atypical chest pain initially are ultimately diagnosed as acute coronary syndrome. A blood biomarker test would be useful to sort out these patients. Although a number of protein biomarkers of inflammation have been discovered, their use in outpatient setting has not been investigated adequately. Only C-reactive protein has been studied sufficiently for analysis of data. However, the threshold for a positive C-reactive protein remains unknown. Published evidence is not yet sufficient to support the routine use of new protein markers in screening for ACS in the emergency department setting.

In spite of the limitation, the most frequently used biomarker in the emergency department (ED) continues to be cardiac troponin (Tn). Other markers that have been used because of the need in the ED for rapid triage have been myoglobin and FABP. In addition, some centers still prefer less sensitive and less specific markers such as CK-MB. More recently, a push has occurred to develop markers of ischemia, such as ischemia modified albumin (IMA), to determine which patients have ischemia, even in the absence of cardiac injury.

Markers of ischemia are useful, but at present, despite some enthusiasm, are not ready for routine use (Jaffe 2005). Before describing the recommendations for clinical use of biomarkers in the ED, a basic understanding of some of the science and measurement issues related to these analytes is helpful.

## *Use of Biomarkers for Prognosis of Recurrent Atrial Fibrillation*

Plasma concentrations of three specific cardiac hsTnT, NT-proBNP, and mid-regional proANP (MR-proANP) and three stable fragments of vasoactive peptides (mid-regional proadrenomedullin [MR-proADM], copeptin [CT-proAVP], and CT-proendothelin-1 [CT-proET1]), were measured at baseline and after 6 and 12 months in patients enrolled in the GISSI-AF (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico-atrial fibrillation) study, a prospective randomized trial to determine the effect of valsartan to reduce the recurrence of AF (Latini et al. 2010). Despite low baseline levels, higher concentrations of hsTnT, MR-proANP, NT-proBNP, and CT-proET independently predicted higher risk of a first recurrence of AF. It was concluded that circulating biomarkers of cardiomyocyte injury are related to recurrence of AF in patients in sinus rhythm with a history of recent AF.

## *Use of Multiple Biomarkers for Monitoring of Cardiovascular Disease*

Currently, screening for cardiovascular disease is primarily accomplished through a series of blood tests that determine levels of a few biomarkers. Methods of cardiovascular disease diagnosis are based on the fact that cardiac marker proteins are released into the blood in large quantities at different rates during cardiac injuries. Traditional methods, such as ELISA, can only measure one cardiac marker at a time. Nanocheck™ AMI (Nano-Ditech Corporation) provides qualitative measurements of three different well-known cardiac makers, cTnI, CK-MB, and myoglobin, simultaneously or separately.

The Evidence Investigator™ (Randox Laboratories) is a research and clinical biochip reader dedicated to multi-analyte analysis portfolio of biochip assays. It is a compact bench-top biochip system comprising a super cooled charge coupled device camera and unique image processing software. It offers a wide-ranging test and "in development" menu for protein and DNA biochips for detection of biomarkers. Rather than having to divide and separately test a patient sample to obtain each test result, evidence investigator™ offers a means for simultaneous testing of a sample, and thus provides a more complete diagnostic profile for each patient. Diagnostic panels are available for several diseases. For example, cardiac array test menu consists of troponin I, myoglobin, CK-MB, carbonic anhydrase III, glycogen phosphorylase BB, and fatty acid-binding protein. All six tests are performed

simultaneously on a single biochip using just one assay diluent, one panel-specific conjugate solution, and one signal reagent.

Few investigations have evaluated the incremental usefulness of multiple biomarkers from distinct biologic pathways for predicting the risk of cardiovascular events. One study measured 10 biomarkers in 3,209 participants attending a routine examination cycle of the Framingham Heart Study: the levels of CRP, BNP, NT ProBNP, aldosterone, renin, fibrinogen, D-dimer, plasminogen-activator inhibitor type 1, and homocysteine; and the urinary albumin-to-creatinine ratio (Wang et al. 2006). The following biomarkers most strongly predicted the risk of death: BNP level, CRP level, the urinary albumin-to-creatinine ratio, homocysteine level, and renin level. The biomarkers that most strongly predicted major cardiovascular events were BNP level and the urinary albumin-to-creatinine ratio. It was concluded that for assessing risk in individual persons, the use of the 10 contemporary biomarkers that were studied adds only moderately to standard risk factors. Because the novel biomarkers do not meaningfully reduce misclassification by traditional risk scoring, they should not be used in basic risk-factor assessment. However, the current data do not rule out a role for biomarkers such as CRP in improving risk prediction in selected patients. Although these biomarkers made a statistically significant contribution to risk prediction, they had limited value for risk stratification of individual patients. The appropriate role of these biomarkers in the clinical care of patients remains to be determined.

## *Use of Biomarkers in the Management of Peripheral Arterial Disease*

Peripheral arterial disease (PAD) shares some of the other risk factors of cardiovascular disease such as inflammation, atherosclerosis, and hypercholesterolemia. CRP is also considered a biomarker of PAD. However, the phase III SIMPADICO trial of its Celacade™ (Vasogen Inc) in PAD did not reach the primary endpoint of change in maximal treadmill walking distance, although it significantly reduced hs-CRP, a prespecified endpoint and a widely recognized marker of systemic inflammation associated with increased cardiovascular risk, including heart failure, stroke, and heart attack.

## *Use of Biomarkers in the Management of Hypertension*

Traditionally, blood pressure measurements have been considered as a marker of hypertension and response to therapy. Some of biomarkers of cardiovascular disease are now being measured as a guide to the treatment of hypertension. Diovan® (valsartan) lowered the level of the inflammatory marker high sensitivity C-reactive protein (hsCRP), independently of its established efficacy in lowering blood pressure,

according to a study by Novartis. The study also showed that Diovan and Co-Diovan, including two new high doses recently approved by the FDA, helped a significant number of hard-to-treat patients with moderate to severe high blood pressure quickly achieve blood pressure goals in as little as 2 weeks.

## Future Prospects for Cardiovascular Biomarkers

### *Cardiovascular Biomarker Consortium*

In 2006, the NHLBI of the NIH started to work with its Framingham Heart Study (FHS) to create a biomarker consortium that will research markers associated with cardiovascular disease. The initiative is developing new diagnostics to identify high-risk individuals, and will focus on risk factors for CVD such as atherosclerosis, obesity, insulin resistance, hypertension, and metabolic syndrome. NHLBI will use information from 7,000 FHS subjects whose CVD risk factors are already known in order to study 150 or more "evolving or novel" biomarkers found in serum, plasma, and urine. Two major aims for the biomarker consortium are:

1. It hopes to "identify the biochemical signature" of atherosclerosis by looking at aortic and coronary calcification, aortic plaque burden, carotid intimal-medial thickness, clinical atherosclerotic CVD, and the balance between calcification of the arteries and bone demineralization.
2. It aims to identify the biochemical signature of metabolic syndrome by studying blood pressure, obesity, visceral adiposity, dyslipidemia, impaired fasting glucose, diabetes, and insulin resistance.

The NHLBI will choose the biomarkers for the study by reviewing biomarkers of atherosclerosis and metabolic syndrome, and by studying genes linked to the two syndromes and those showing links with "phenotypes of interest." During the study, new quantitative tests will be developed that can measure circulating biomarker levels by using antibody sandwich assays and proteomic approaches that work with high-throughput applications. The study will focus on pathways: adhesion/chemoattraction, adipokines, cytokines, growth factors, heart shock proteins, inflammation, lipoproteins, neurohormones, thrombosis/fibrinolysis, and vascular calcification.

### *Systems Approach to Biomarker Research in Cardiovascular Disease*

Systems Approach to Biomarker Research in Cardiovascular Disease (SABRe CVD) will identify and validate new biomarkers such as proteins or molecules

in the blood for heart disease. The landmark FHS is launching a major initiative to discover risk factors and biomarkers that could lead to new blood tests to identify individuals at high risk of heart disease and stroke. It is funded by NHLBI and conducted in collaboration with Boston University School of Medicine and School of Public Health. A public–private partnership has been established to enable researchers to apply cutting-edge technology to stored blood samples from thousands of FHS participants. An important component of the biomarker research will be conducted under a 5-year CRADA with BG Medicine, which has developed patented technology to detect and validate subtle biological changes at the molecular level. In 2010, Sigma-Aldrich joined this project to discover biomarkers for atherosclerosis CVD in plasma samples. Sigma will develop antibody reagents for each identified target biomarker and incorporate the reagents into a multiplexed, high-throughput platform to measure proteins of interest.

Researchers from participating institutions will study about 1,000 blood biomarkers. Frozen blood samples, imaging studies, and other medical test results gathered over the years from more than 7,000 FHS participants of diverse ages will be analyzed to identify which blood biomarkers are associated with heart disease, metabolic syndrome, and related risk factors. Researchers will use only materials from participants who have consented to sharing their specimens and data with commercial sector scientists, and all shared information will be de-identified to protect participants' privacy.

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# Chapter 5
# Molecular Diagnosis of Cardiovascular Disorders

## Introduction

Symptoms of cardiovascular diseases can often be confused with other pathologic conditions and it is important that an accurate and expeditious diagnostic system is available to help physicians make a rapid diagnosis. The classical methods of cardiac diagnosis by auscultation, clinical acumen, and electrocardiography (ECG) are now supplemented by echocardiography, cardiac molecular imaging, isotope scintigraphy, and refined techniques of coronary catheterization, which can be combined with therapeutic interventions. Biotechnology is also making important contributions to cardiovascular diagnostics and some examples are given in this chapter.

## *Basics of Molecular Diagnosis*

Basic technologies of molecular diagnostics are the Southern blot, DNA probes, pulsed field gel electrophoresis, and polymerase chain reaction (PCR). These technologies, their innovations, and non-PCR alternatives are described in detail in a special report on molecular diagnostics (Jain 2011a). PCR is a method of nucleic acid analysis for producing large amounts of a specific DNA fragment of a defined sequence and length from a small amount of a complex template. It can selectively amplify a single molecule of DNA or RNA several millionfold in a few hours. Use of this technology enables the detection and analysis of specific gene sequences in a patient's sample without cloning. Analyses can be performed on even a few cells from body fluids or in a drop of blood. Thus, PCR eliminates the need to prepare large amounts of DNA from tissue samples. PCR has revolutionized molecular diagnostics. Apart from laboratory diagnosis, it has affected genomics and biotechnology as well.

PCR is based on the enzymatic amplification of a fragment of DNA that is flanked by two "primers" – short oligonucleotides that hybridize to the opposite strands of the target sequence and then prime synthesis of the complementary DNA sequence by DNA polymerase (an enzyme). The chain reaction is a three-step

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process – denaturation, annealing, and extension that is repeated in several cycles. At each stage of the process, the number of copies is doubled – from two, to four, to eight, and so on. The reactions are controlled by changing the temperature using a special heat-stable Taq polymerase. After 20 cycles, roughly 1 million copies exist, or enough material to detect the desired DNA by conventional means such as color reaction.

RNA can also be studied by making a DNA copy of the RNA using the enzyme reverse transcriptase. Such an approach enables the study of mRNA in cells that use the molecule to synthesize specific proteins or the detection of the genome of RNA viruses. PCR has been fully automated via use of thermal cycling. It is a fast, sensitive, and specific test with applications in diagnosis of various diseases.

Development of microarray/technology has further advanced the applications of molecular diagnostics (Jain 2011b). It will facilitate the point-of-care (POC) diagnosis of genetic cardiovascular disorders and enable the development of personalized cardiology.

Biosensors used in molecular diagnostics are based on antibodies, enzymes, ion channels, or nucleic acids. Biosensors incorporate a biological sensing element that converts a biological event into an electrical signal that can be processed. Almost all analytical systems combine sensing (i.e., detection) and transducing components; the distinct feature of biosensors is that the two functions are coupled in a single physical entity. A biosensor's input is a specific biological event (e.g., binding of an antigen to an antibody). Its output is a measurable signal that corresponds to the input. A biosensor's biological component provides specificity, the ability to selectively recognize one type event. Its transducer confers sensitivity, the ability to transform the very low energy of the biological event into a measurable signal.

## *Molecular Imaging of Cardiovascular Disorders*

Technologies encompassed within molecular imaging include optical, magnetic resonance imaging (MRI), and nuclear medicine techniques, which are refinements of conventional imaging techniques such as positron emission tomography (PET). Molecular single photon emission CT (SPECT) and PET imaging strategies can be used for the evaluation of cardiovascular disease, including investigation of myocardial metabolism and neurohumoral activity of the heart as well as for evaluation of processes such as atherosclerosis, ventricular remodeling after myocardial infarction, and ischemia-associated angiogenesis at molecular level (Dobrucki and Sinusas 2010).

Molecular imaging provides in vivo information in contrast to the in vitro diagnostics. Cardiovascular magnetic resonance (CMR) molecular imaging can identify and map the expression of important biomarkers on a cellular scale using contrast agents that are specifically targeted to the biochemical signatures of disease and are capable of generating sufficient image contrast. Contrast agents may help to integrate diagnosis with therapy as they can be designed to be sensitive to

important physiological factors, such as pH, temperature, or oxygenation and carry therapeutics as well. Several molecular imaging quantification techniques have been described that include measurement of signal changes, calculation of the area of contrast enhancement, mapping of relaxation time changes, or direct detection of contrast agents through multinuclear imaging or spectroscopy (Winter et al. 2010). CMR molecular imaging can be used for early detection of therapeutic response to drugs, localization of ruptured atherosclerotic plaques, stratification of patients based on disease biomarkers, tissue-specific drug delivery, confirmation and quantification of end-organ drug uptake, and noninvasive monitoring of disease recurrence.

## Genetic Cardiovascular Disorders

Several cardiovascular diseases have been recognized to have a genetic component; indeed, a family history of heart disease has always attracted the physician's attention. In recent years, molecular genetics has contributed to the development of molecular cardiology, opening up some new pathways to the diagnosis, prevention, and treatment of some cardiovascular diseases. Genetic approaches have succeeded in defining the molecular basis of an increasing array of heart diseases, such as hypertrophic cardiomyopathy and the long-QT syndrome, associated with serious arrhythmias. Some of the genes that cause cardiovascular diseases are described in Chap. 9.

## Coronary Heart Disease

Coronary heart disease (CHD) is associated with various disturbances in the coronary artery circulation. It may involve stenosis or narrowing with reduced blood flow or complete occlusion of the coronary artery or arteries, resulting in myocardial infarction. Linkages have been demonstrated between DNA markers and both risk factors and certain overt CHD. Many of these correlations involve not merely one gene, however, but entire chromosome regions containing hundreds of genes. Such vague relationships may not help to establish a diagnosis as required by either epidemiology or molecular genetics.

Genome-wide screening of affected siblings has now emerged as a promising route to identifying new genes or confirming the roles of established genes for CHD. For each tentative new locus, siblings with premature-onset CHD are compared to see whether they prove identical for one or both parental alleles more often than would be expected from Mendelian segregation. New common polymorphisms detected in such screening, however, must be proved to be functional before they can be confirmed as genetic risk factors for CHD.

Development and progression of atherosclerosis involves recruitment and binding of circulating leukocytes to areas of inflammation within the vascular

endothelium mediated by cellular adhesion molecules. A polymorphism in the endothelial-leukocyte adhesion molecule 1 (E-selectin) gene has been implicated in the early onset of atherosclerotic disease because it profoundly affects binding specificity, resulting in a significant increase in cellular adhesion. There is a relation between the E-selectin S128R polymorphism and coronary artery calcification, a biomarker of atherosclerosis, in asymptomatic women 50 years of age or younger, who have low levels of traditional risk factors and reduced adhesion molecule expression due to the presence of high levels of endogenous hormones.

Although several risk factors for CHD have been identified, such as high cholesterol and smoking, they explain only about one-half of first heart attacks. It is now known that inflammation in the arteries is a major part of the biological process that leads to atherosclerosis and the resulting MIs and strokes. IL-1 gene cluster, located on human chromosome 2, greatly influences how the body responds to inflammation. Expression of IL-1 is likely to have a significant role in signaling artery wall damage.

Interleukin Genetics is using IL-1 gene expression for investigating new opportunities to identify individuals at high risk for CHD as well as providing new targets for drugs to prevent heart disease and strokes.

Corus CAD test (CardioDX) measures the expression of 23 genes to identify patients at risk for CHD. Mitochondrial DNA (mtDNA) damage in blood cells is a specific indicator of oxidative stress, which is a risk factor for CHD. Transgenomic is developing a qPCR-based technology, licensed from Clayton Foundation for Research (Houston, TX), to detect mtDNA damage for predicting CHD. This technology is based on US Patent #6322974 of 2001, which states that mtDNA damage occurs early in atherosclerosis and is the basis for methods to predict CHD based upon the amount of mtDNA damage.

## Cardiomyopathy

Cardiomyopathy encompasses heart muscle diseases of unknown cause. Two main types have been identified according to the anatomical change: hypertrophic and dilated.

### *Familial Hypertrophic Cardiomyopathy*

Familial hypertrophic cardiomyopathy (FHC) is the first inherited primary cardiomyopathy for which genetic studies have been conducted. This autosomal dominant inherited disease is an important cause of sudden death in healthy young individuals, such as athletes.

The first chromosomal locus has been mapped on chromosome 14 at q11-q12, where the putative gene encodes the beta-myosin heavy chain. The challenge for

the future lies in identifying other genes causing FHC, clarifying the relationships between various gene defects and the development of the disease, and using genetic data for diagnostic and possibly therapeutic purposes. Mutations for alpha tropomyosin are found in 3% and mutations in cardiac troponin T in 15% of cases of FHC. The clinical signs are mild but sudden death may occur. Genetic testing is especially important in this group.

### *Idiopathic Dilated Cardiomyopathy*

Recent clinical surveys indicate that genetic factors play a major role in the pathogenesis of idiopathic dilated cardiomyopathy (IDC). In families with matrilineal transmission, the disease gene can be linked to mtDNA alterations. Autosomal familial IDC, the most frequent form, is currently under active investigation. Other candidate genes are dystrophin and actin.

## Cardiac Arrhythmias

In victims of sudden cardiac death, the heart may appear to be grossly and histologically normal. A molecular autopsy including sampling for genetic testing may reveal evidence of inherited cardiac rhythm disorders, for example, those due to ion channel diseases such as Brugada syndrome (Basso et al. 2010). Investigation of the molecular mechanisms that lead from the gene defect to the clinical phenotype may lead to improvements in the prevention of sudden cardiac death in the young and screening of their relatives (Shephard and Semsarian 2009). The way new knowledge can be applied to managing patients and to the development of syndrome-specific antiarrhythmic strategies is evolving rapidly because of the recent advances in genetics. A few examples are briefly described here.

### *Long Q-T Syndrome*

Long Q-T syndrome is an inherited form of ventricular arrhythmia in which the interval between the Q and the T waves is longer than normal. This disease reflects a defect in the electrical properties of the cardiac muscle, which predisposes the patient to life-threatening ventricular fibrillation after stress. Five genes have been identified where the mutations are associated with this disorder. These genes encode cardiac potassium ion channels and support the hypothesis that the LQT syndrome results from delayed myocellular repolarization. The diagnosis of long QT syndrome and other channelopathies by an electrocardiogram is often difficult and may be missed, which leaves a patient at risk for sudden cardiac death. FAMILION™ (Transgenomic) is the first commercially available, comprehensive

genetic test for cardiac channelopathies and cardiomyopathies that can guide the physician in determining the best treatment options for those who are genetically predisposed to potentially fatal cardiac arrhythmias caused by long QT syndrome and related cardiac ion channel diseases. The test examines five cardiac ion channel genes for a mutation that is likely to cause long QT syndrome. If a genetic mutation is detected, its type and location can assist the physician in making treatment selections that could include life-style modification, prescription or avoidance of specific classes of drugs or the implantation of a defibrillator. A patient's family members also benefit from the test because it can identify if they inherited the same mutation as the initially symptomatic patient and may be at risk of a potentially fatal arrhythmia. These relatives often have ambiguous findings on an ECG, while the results of the FAMILION™ test can answer whether or not they carry the familial mutation.

## *Familial Atrial Fibrillation*

Familial atrial fibrillation, the most common sustained cardiac rhythm disturbance, affects more than 2 million people in the USA, with an overall prevalence of 0.89%. The prevalence of this disease rises to 5.9% in individuals over the age of 65, and it accounts for one third of all strokes in these patients. The clinical manifestations of familial atrial fibrillation range from palpitations to heart failure. The molecular basis of this condition is unknown, and physicians typically resort to palliative therapy in an effort to control ventricular rate and prevent systemic emboli.

A genetic locus for familial atrial fibrillation has been identified at 10q22–q24 by the time – and cost-effective method of pooling DNA of affected and unaffected family members. Researchers speculate, however, that the basis for familial atrial fibrillation lies in abnormal atrial triggering because the genes that encode adrenergic receptors are located at the mutation site on chromosome 10q. Small molecular defects in DNA can change the electrophysiological properties of the atria; these alterations may, in turn, create a substrate for chronic atrial fibrillation.

## *Idiopathic Ventricular Fibrillation*

Ventricular fibrillation is the cause of more than 300,000 sudden deaths each year in the USA. In about 12% of the cases, there is no demonstrable cardiac or noncardiac cause and these cases are classified as idiopathic ventricular fibrillation. A group of these patients present with characteristic electrocardiographic changes; this category is also called Brugada Syndrome. Mutations in the sodium channel gene SCN5A contribute to the risk of developing idiopathic ventricular fibrillation.

## Early Detection of Congestive Heart Failure

Early detection of CHF is an important factor in reducing mortality and morbidity associated with the condition. However, diagnosis of CHF is a complex and often expensive process. The symptoms of CHF are nonspecific and are sometimes confused with those of other conditions such as chronic obstructive pulmonary disease. Echocardiography, the gold standard for diagnosis of left ventricular dysfunction, is expensive and not always easily accessible. Other items on the cardiovascular panel are CK-MB, myoglobin, troponin I, and homocysteine. These are expanded by the BNP (B-type natriuretic peptide) assay. The BNP molecule is physiologically active and measures the direct biologic response to cardiovascular stress providing a direct reflection of the patient's current status. This allows more timely treatment and patient management. Recent research has shown that elevated levels of BNP indicate the presence of CHF, thus providing physicians with an important diagnostic tool in the early detection and treatment.

Triage BNP Test (Biosite Inc), based on protein chip technology, is a quantitative test for measurement of BNP in CHF. ADVIA Centaur® BNP assay (Bayer Diagnostics) is a fully automated test that provides results in minutes, requiring less hands on labor while reducing human error associated with manual testing. The assay is currently available only outside the USA. Roche Diagnostics's Elecsys® proBNP is an automated immunoassay for diagnosis of CHF by detecting the level of the NT-proBNP peptide. It was approved by the FDA in 2003 for risk stratification in CHF and acute coronary syndrome.

## Genetic Testing in Hypertension

The angiotensinogen (AGT) gene has been implicated as a candidate gene of high blood pressure. AGT gene and some of its variants represent the best examples of genetic influences that are involved in the determination of essential hypertension and associated cardiovascular diseases. However, because the variants of the AGT gene are point mutations, it is difficult to detect them in large-scale population studies.

CardiaRisk (Myriad Genetic Laboratories) is a genetic analysis of the AGT gene, which helps identify individuals with a particular form of hypertension and to individualize therapy by analyzing genetic factors that influence each patient's physiology and responsiveness to various interventions. It identifies those patients who may benefit most from a low-salt diet and who also may have a greater risk of cardiovascular diseases, such as coronary heart disease and myocardial infarction. The test may also be useful in the T235 variant of the AGT gene that has been associated with an increased risk of coronary heart disease and certain forms of hypertension.

## Gene Mutations and Disturbances of Blood Lipids

Hundreds of mutations have been reported in genes encoding apolipoproteins, lipolytic enzymes, and lipoprotein receptors. Such defects can cause serious perturbations in blood lipid concentrations.

### *Familial Dyslipoproteinemias*

These have been characterized at the molecular level. For instance, functional polymorphism of apolipoprotein E (encoded on chromosome 19q13) can be assessed by PCR. In the normal population, polymorphism of E2, E3, and E4 exerts a substantial effect on the normal variations in the plasma lipid concentrations, accounting for 16% of the genetic variation in cholesterol concentration. An age-related increase of E2 allele and a decrease of E4 allele, for example, may contribute to the increased number of deaths from myocardial infarction. Homozygosity of the E2 allele, occurring in association with diabetes mellitus, hypothyroidism, or other genetic defect of lipid metabolism, can lead to dysbetalipoproteinemia (type III hyperlipidemia); this condition is characterized by profound hypertriglyceridemia, moderate hypercholesterolemia, and premature CHD.

Lipoprotein (a) may be a monogenic risk factor for CHD, accounting for a major portion of the familial predisposition to CHD that cannot be explained by other factors. The Apo (a) gene locus determines more than 90% of the variations in plasma lipoprotein (a) concentration.

### *Hypercholesterolemia*

Individuals with familial hypercholesterolemia (FH) manifest defective catabolism of low-density lipoprotein (LDL), which increases plasma circulating LDL cholesterol levels two- to threefold. This rise in LDL levels may cause cholesterol to be deposited in the arterial wall, leading to premature atherosclerosis and CHD. Predictive identification of at-risk individuals is important because prophylactic treatment with cholesterol-lowering drugs can reduce morbidity and mortality. One of the most common single-gene disorders, heterozygous FH, affects approximately 1 in 500 individuals.

Mutations in the LDL gene are a major cause of FH, and more than 300 different point mutations and short deletions in this gene have been characterized to date. One such mutation found in the Finnish population (FH-North Karelia) deletes seven nucleotides from exon 6 of the LDL receptor gene. PCR amplification of DNA samples from patients with this condition prompted the formation of DNA heteroduplexes that markedly improved mutation detection. Multiplex PCR,

combined into an oligonucleotide ligation assay, has been used for the direct assay of prevalent mutations in FH. Detection is done by standard multicolor fluorescence technology, chemically introduced into oligonucleotides.

Some patients with a family history of hypercholesterolemia have a defect in the gene for apolipoprotein B, the component of low density lipoprotein that binds the receptor.10 This ligand defect is called familial defective apoB. One common mutation of the gene is R3500Q, which occurs in 1/700–1/1,000 people in central and western Europe. It causes severe hypercholesterolemia and increases the risk of ischemic heart disease. A simple genetic test for the R3500Q mutation is available, and some family members of known probands ask to be tested for the gene. The clinical significance of a positive test may be hard to determine, however, especially if the individual concerned has a normal cholesterol level.

## Gene Mutations Associated with Thrombotic Disorders

A number of thrombotic disorders cause cardiovascular disease as well as stroke (see the following section on neurological disorders). Venous thrombosis has an annual incidence of 1/1,000 in the general population and is associated with significant morbidity and mortality. Several genetic variants have been identified that are associated with an increased risk of venous thrombosis, including a recently discovered mutation in the prothrombin gene. Individuals who die suddenly from pulmonary embolism are not often affected by prothrombin or factor V gene mutations. Factor V Leiden mutation, however, is the most common genetic cause of idiopathic thrombotic disorders.

### *Factor V Leiden Mutation*

A mutation in the procoagulant protein Factor V (Factor V Leiden) causes it to be relatively resistant to degradation by activated protein C (APC), resulting in a thrombotic tendency. The mutation is a guanine-to-adenine substitution at nucleotide 1,651 that results in a glutamine-to-arginine substitution at position 506 (R506Q). This is a clinically significant mutation, since it is relatively common (found in 3–6% of Caucasian subjects) and has been shown to be associated with venous thrombosis and stroke. There is justification for a rapid method of screening for this mutation in large populations. THRIMBOGENE, a commercial PCR-based test for the detection of factor V Leiden mutation, is available from Athena Diagnostics for identifying a hypercoagulable state in stroke. Although PCR-RFLP is a standardized assay with relatively reliable performance, the technique is cumbersome and labor intensive, limiting its general applicability in diagnostic laboratories. As a result, many laboratories use a functional clotting assay as a

screening or diagnostic test for Factor V Leiden. Although the clotting assay compares well with PCR-RFLP, follow-up molecular testing is recommended in samples showing borderline or abnormal results, to rule out other causes of functional APC resistance and to accurately distinguish heterozygotes from homozygotes. The latter is a critical distinction, as the thrombotic risk for homozygotes is about ten times higher than the already elevated risk in Factor V Leiden heterozygotes.

PCR-based methods for the detection of factor V Leiden mutation show 100% concordance in the results in that all of the laboratories could reliably identify the single base pair substitution. Invader assay (Hologic Inc) amplifies linearly without the need for PCR or gel electrophoresis and has been shown to detect this mutation reliably in a THROMBO study. An ELISA-like assay has been developed by Exiqon to detect the mutation in PCR-amplified genomic DNA using LNAs. In 2003, FDA approved the first DNA-based laboratory tests for this mutation – Factor V Leiden and the Factor II (prothrombin) G20210A kit.

### *Pulmonary Embolism*

Pulmonary embolism (PE) is a blockage of one or more of the pulmonary arteries by blood clot, which is a common and highly lethal condition that is a leading cause of death in all age groups. PE is the third most common cause of death in the USA with at least 650,000 cases occurring annually. Currently, more cases of PE are missed than are actually diagnosed because of vague and nonspecific symptoms. It is the first or second most common cause of unexpected death in most age groups. The highest incidence of unrecognized PE occurs in hospitalized patients. Autopsy results show as many as 60% of patients dying in the hospital have a PE, but the diagnosis is missed in about 70% of the cases.

Triage latex-agglutination D-Dimer Test (Biosite Inc) is a portable and easy-to-use, point-of-care (POC) diagnostic test that can be used in the emergency department or at the patients' bedside. A POC clinical protocol for the evaluation of PE using D-Dimer test doubled the number of patients evaluated for PE and decreased length of stay in the emergency department, without increasing the need for vascular imaging. This test can play an important role in helping emergency department physicians rapidly evaluate patients with suspected pulmonary embolism, which should result in better patient management.

## Companies Involved in Cardiovascular Molecular Diagnosis

A selection of companies developing molecular diagnostics for cardiovascular diseases is shown in Table 5.1.

**Table 5.1** A selection of companies involved in molecular diagnostics for cardiovascular diseases

| Test/company | Method/application | Status/comments |
|---|---|---|
| Cardiac Array Evidence Investigator™/Randox Laboratories | Biochip technology to simultaneously measure four cardiac biomarkers of patients with chest pain and suspected CAD with results in <30 min | Marketed for research and clinical laboratories |
| CardiaRisk/Myriad Genetic Laboratories | Genetic analysis of the AGT gene to identify analyzing genetic factors that influence a patient's hypertension and responsiveness to various interventions to individualize therapy | Marketed |
| Corus CAD/CardioDX | Blood text for measuring expression of 23 genes to identify patients at risk for CAD | Marketed |
| Elecsys® proBNP/Roche Diagnostics | Automated immunoassay for diagnosis of CHF by detecting the level of the NT-proBNP | Approved by the FDA |
| FAMILION™/ Transgenomic | Comprehensive genetic test for cardiac channelopathies and cardiomyopathies | Approved and marketed |
| Assays for mutations in Factor V (Leiden) and Factor II (prothrombin) genes/Hologic Inc | Mutations in these genes are associated with deep vein thrombosis and pulmonary embolism | Approved by the FDA |
| IL-1 gene expression/ Interleukin Genetics | To identify patients at high risk for heart disease<br>To provide drug targets for preventing heart disease | Exploratory research |
| Nanocheck™/Nano-Ditech Corporation | Provides qualitative measurements of three different well-known cardiac biomarkers simultaneously or separately for detection of AMI: cTnI, CK-MB and myoglobin | Available in 20 countries round the world |
| qPCR-based for mtDNA/Transgenomics | To detect mtDNA damage for predicting CHD | In development |
| Triage BNP Test/Biosite Inc | Protein chip quantitative fully automated test based on ADVIA Centaur® BNP assay for measurement of BNP in CHF | Available outside of USA |
| Triage latex-agglutination D-Dimer Test/Biosite Inc | Portable and easy-to-use POC test that can be used in the emergency department or at the patients' bedside for detection of pulmonary embolism | Marketed |

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# Chapter 6
# Nanobiotechnology in Cardiovascular Disorders

## Introduction

Nanotechnology (Greek word nano means dwarf) is the creation and utilization of materials, devices, and systems through the control of matter on the nanometer-length scale, that is, at the level of atoms, molecules, and supramolecular structures. Nanotechnology, as defined by the National Nanotechnology Initiative (http://www.nano.gov/), is the understanding and control of matter at dimensions of roughly 1–100 nm, where unique phenomena enable novel applications (Jain 2011). Encompassing nanoscale science, engineering and technology, nanotechnology involves imaging, measuring, modeling, and manipulating matter at this length scale. It is the popular term for the construction and utilization of functional structures with at least one characteristic dimension measured in nanometers – a nanometer is one billionth of a meter ($10^{-9}$ m). Given the inherent nanoscale functional components of living cells, it was inevitable that nanotechnology will be applied in biotechnology giving rise to the term nanobiotechnology. Nanomedicine is defined as the application of nanobiotechnology to medicine, and some of these applications are shown in Table 6.1 (Jain 2008).

Nanocardiology is the application of nanobiotechnology to cardiovascular diseases. Recent rapid advances in nanotechnology and nanoscience offer a wealth of new opportunities for diagnosis and therapy of cardiovascular and pulmonary diseases. As far back as 2003, the National Heart, Lung, and Blood Institute of USA convened a Working Group on Nanotechnology for translational applications to heart, lung, blood disorders, and cardiovascular complications of sleep apnea to solve clinical problems.

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**Table 6.1** Nanomedicine in the twenty-first century

| |
|---|
| *Nanodiagnostics* |
| Molecular diagnostics |
| Nanoendoscopy |
| Nanoimaging |
| *Nanotechnology-based drugs* |
| Drugs with improved methods of delivery |
| *Regenerative medicine* |
| Tissue engineering with nanotechnology |
| *Transplantation medicine* |
| Exosomes from donor dendritic cells for drug-free organ transplants |
| *Nanorobotic treatments* |
| Vascular surgery by nanorobots introduced into the vascular system |
| Nanorobots for detection and destruction of cancer |
| *Implants* |
| Bioimplantable sensors that bridge the gap between electronic and neurological circuitry |
| Durable rejection-resistant artificial tissues and organs |
| Implantations of nanocoated stents in coronary arteries to elute drugs and to prevent reocclusion |
| Implantation of nanopumps for drug delivery |
| *Minimally invasive surgery using catheters* |
| Miniaturized nanosensors implanted in catheters to provide real-time data to surgeons |
| NanoSurgery by integration of nanoparticles and external energy |



## Nanotechnology-Based Cardiovascular Diagnosis

### *Nanobiotechnology for Molecular Diagnostics*

Numerous nanodevices and nanosystems for sequencing single molecules of DNA are feasible. It seems quite likely that there will be numerous applications of inorganic nanostructures in biology and medicine as markers. Given the inherent nanoscale of receptors, pores, and other functional components of living cells, the detailed monitoring and analysis of these components will be made possible by the development of a new class of nanoscale probes. Biological tests measuring the presence or activity of selected substances become quicker, more sensitive, and more flexible when certain nanoscale particles are put to work as tags or labels. Nanotechnology will improve the sensitivity and integration of analytical methods to yield a more coherent evaluation of life processes. Potential applications of nanotechnology in molecular diagnostics are self-diagnostics for use in the home and sensors for labs-on-a-chip (Jain 2007). A classification of nanotechnologies with potential applications in molecular diagnostics is shown in Table 6.2. Many of these are used for both nucleic acids and proteins.

**Table 6.2** Nanotechnologies with potential applications in molecular diagnostics

| |
|---|
| *Nanotechnology on a chip* |
| Microfluidic chips for nanolitre volumes: nanochip |
| Optical readout of nanoparticle labels |
| Nanoarrays |
| Protein nanoarrays |
| *Nanoparticle technologies* |
| Gold particles |
| Nanobarcodes |
| Magnetic nanoparticles: ferrofluids |
| Quantum dot technology |
| Nanoparticle probes |
| *Nanowires* |
| *Nanopore technology* |
| *Cantilever arrays* |
| *DNA nanomachines for molecular diagnostics* |
| *Nanosensors* |
| Living spores as nanodetectors |
| Quartz nanobalance DNA sensor |
| PEBBLE (Probes Encapsulated by Biologically Localized Embedding) nanosensors |
| Nanosensor glucose monitor |
| *Nanochip-based single-molecular interaction force assays* |
| *Resonance light scattering technology* |



## *Nanosensors*

Nanomaterials are exquisitely sensitive chemical and biological sensors. Since their surface properties are easily modified, nanowires can be decorated with virtually any potential chemical or biological molecular recognition unit, making the wires themselves analyte independent. The nanomaterials transduce the chemical binding event on their surface into a change in conductance of the nanowire in an extremely sensitive, real time, and quantitative fashion. Boron-doped silicon nanowires (SiNWs) have been used to create highly sensitive, real-time electrically based sensors for biological and chemical species. Biotin-modified SiNWs were used to detect streptavidin down to at least a picomolar concentration range. The small size and capability of these semiconductor nanowires for sensitive, label-free, real-time detection of a wide range of chemical and biological species could be exploited in array-based screening and in vivo diagnostics.

The sensors can be electronically gated to respond to the binding of a single molecule. Prototype sensors have demonstrated detection of nucleic acids, proteins, and ions. These sensors can operate in the liquid or gas phase, opening up an enormous variety of downstream applications. The detection schemes use inexpensive low voltage measurement schemes and detect binding events directly so there is no need for costly, complicated, and time-consuming labeling chemistries such as fluorescent

dyes or the use of bulky and expensive optical detection systems. As a result, these sensors are inexpensive to manufacture and portable. It may even be possible to develop implantable detection and monitoring devices based on these detectors.

## *Use of Magnetic Nanoparticles as MRI Contrast Agents for Cardiac Disorders*

Magnetic nanoparticles have been used as contrast agent for MRI and have refined molecular imaging. Targeted imaging of vascular inflammation or thrombosis may enable improved risk assessment of atherosclerosis by detecting plaques at high risk of acute complications (Saraste et al. 2009). Cell death in the heart can be imaged in vivo by using annexin-labeled magnetic nanoparticles, particularly AnxCLIO-Cy5.5 (Chen et al. 2011). Experimental studies have shown the feasibility of combination of diagnosis and therapy using magnetic nanoparticles. Magnetic nanoparticles, conjugated with plasmid DNA expressing enhanced green fluorescent protein and coated with chitosan, were injected into mice through the tail vein and directed to the heart by means of an external magnet without the need to functionalize the nanoparticles, and their location was confirmed by fluorescent imaging (Kumar et al. 2010). This approach requires further investigations before clinical applications can be considered.

## *Use of Perfluorocarbon Nanoparticles in Cardiovascular Disorders*

Perfluorocarbon (PFC) nanoparticles provide an opportunity for combining molecular imaging and local drug delivery in cardiovascular disorders. Ligands such as MAbs and peptides can be cross-linked to the outer surface of PFCs to enable active targeting to biomarkers expressed within the vasculature. PFC nanoparticles are naturally constrained by size to the circulation, which minimizes unintended binding to extravascular, nontarget tissues expressing similar epitopes. Moreover, their prolonged circulatory half-life of approximately 5 h allows saturation of receptors without addition of PEG or lipid surfactant polymerization. The utility of targeted PFC nanoparticles has been demonstrated for a variety of applications in animal models and phantoms, including the diagnosis of ruptured plaque, the quantification, and antiangiogenic treatment of atherosclerotic plaque and the localization and delivery of antirestenotic therapy following angioplasty (Lanza et al. 2006).

## *Cardiac Monitoring in Sleep Apnea*

Because sleep apnea is a cause of irregular heartbeat, hypertension, heart attack, and stroke, it is important that patients be diagnosed and treated before these highly

deleterious sequelae occur. For patients suspected of experiencing sleep apnea, in vivo sensors could constantly monitor blood concentrations of oxygen and cardiac function to detect problems during sleep. In addition, cardio-specific antibodies tagged with nanoparticles may allow doctors to visualize heart movement while a patient experiences sleep apnea to determine both short- and long-term effects of apnea on cardiac function.

## *Detection and Treatment of Atherosclerotic Plaques in the Arteries*

A key feature of the atherosclerotic process is the angiogenic expansion of the vasa vasorum in the adventitia, which extends into the thickening intimal layer of the atheroma in concert with other neovessels originating from the primary arterial lumen. Magnetic resonance molecular imaging of focal angiogenesis with integrin-targeted paramagnetic contrast agents has been reported with PFC nanoparticles and liposomes. Site-targeted PFC nanoparticles also offer the opportunity for local drug delivery in combination with molecular imaging.

The diagnosis and treatment of unstable plaque is an area in which nanotechnology could have an immediate impact. Fibrin-specific PFC nanoparticles may allow the detection and quantification of unstable plaque in susceptible patients, which may be an important feature of future strategies to prevent heart attacks or stroke. Research is under way using probes targeted to plaque components for noninvasive detection of patients at risk. In an extension of this approach, targeted nanoparticles, multifunctional macromolecules, or nanotechnology-based devices could deliver therapy to a specific site, localized drug release being achieved either passively (by proximity alone) or actively (through supply of energy as ultrasound, near-infrared, or magnetic field). Targeted nanoparticles or devices could also stabilize vulnerable plaque by removing material, for example, oxidized low-density lipoproteins. Devices able to attach to unstable plaques and warn patients and emergency medical services of plaque rupture would facilitate timely medical intervention.

## *Monitoring for Disorders of Blood Coagulation*

Patients would benefit greatly from nanotechnological devices that could monitor the body for the onset of thrombotic or hemorrhagic events. Multifunctional devices could detect events, transmit real-time biologic data externally, and deliver anticoagulants or clotting factors to buy critical time.

A gold nanoparticle-based simple assay has been described that enables the visual detection of a protease (Guarise et al. 2006). The method takes advantage of the high molar absorptivity of the plasmon band of gold colloids and is based on the color change of their solution when treated with dithiols. Contrary to the native

ones, cleaved peptides are unable to induce nanoparticles aggregation; hence, the color of the solution does not change. The assay was used to detect two proteases: thrombin (involved in blood coagulation and thrombosis) and lethal factor (an enzyme component of the toxin produced by *Bacillus anthracis*). The sensitivity of this nanoparticle-based assay is in the low nanomolar range.

## Nanotechnology-Based Therapeutics for Cardiovascular Diseases

### *Nanolipoblockers for Atherosclerotic Arterial Plaques*

Nanoscale particles can be synthetically designed to potentially intervene in lipoprotein matrix retention and lipoprotein uptake in cells – processes central to atherosclerosis. These micelles can be engineered to present varying levels of anionic chemistry, which is a key mechanism to induce differential retentivity of low-density lipoproteins (LDLs). Rutgers University scientists have reported on lipoprotein interactions of nanoscale micelles self-assembled from amphiphilic scorpion-like macromolecules based on a lauryl chloride-mucic acid hydrophobic backbone and poly(ethylene glycol) shell. They have used nanoengineered molecules called nanolipoblockers (NLBs) to attack atherosclerotic plaques due to raised levels of LDLs (Chnari et al. 2006). Their approach contrasts with statin drug therapy, which aims to reduce the amount of LDL throughout the body. NLPs compete with oxidized LDLs for a macrophage's attention. The NLBs bind to receptor sites on macrophages, cutting the accumulation of oxidized LDL by as much as 75%.

## Nanotechnology-Based Drug Delivery in Cardiovascular Diseases

Nanobiotechnology provides the following solutions to the problems of drug delivery (Jain 2011):

- Improving solubilization of the drug.
- Using noninvasive routes of administration eliminates the need for administration of drugs by injection.
- Development of novel nanoparticle formulations with improved stabilities and shelf lives.
- Development of nanoparticle formulations for improved absorption of insoluble compounds and macromolecules enable improved bioavailability and release rates, potentially reducing the amount of dose required and increasing safety through reduced side effects.

- Manufacture of nanoparticle formulations with controlled particle sizes, morphology, and surface properties would be more effective and less expensive than other technologies.
- Nanoparticle formulations that can provide sustained release profiles up to 24 h can improve patient compliance with drug regimens.
- Direct coupling of drugs to targeting ligand restricts the coupling capacity to a few drug molecules but coupling of drug carrier nanosystems to ligands allows import of thousands of drug molecules by means of one receptor targeted ligand. Nanosystems offer opportunities to achieve drug targeting with newly discovered disease-specific targets.
- The future of cardiovascular diagnosis already is being impacted by nanosystems that can both diagnose pathology and treat it with targeted delivery systems.
- Controlled delivery of nanoparticles to injured vasculature.
- Nanoparticles for cardiovascular imaging and targeted drug delivery.

The potential dual use of nanoparticles for both imaging and site-targeted delivery of therapeutic agents to cardiovascular disease offers great promise for individualizing therapeutics. Image-based therapeutics with site-selective agents should enable verification that the drug is reaching the intended target and a molecular effect is occurring. Experimental studies have shown that binding of paclitaxel to smooth muscle cells in culture has no effect in altering the growth characteristics of the cells. If paclitaxel-loaded nanoparticles are applied to the cells, however, specific binding elicits a substantial reduction in smooth muscle cell proliferation, indicating that selective targeting may be a requirement for effective drug delivery in this situation. Similar behavior has been demonstrated for doxorubicin-containing particles. Intravenous delivery of fumagillin (an antiangiogenic agent)-loaded nano-particles targeted to $\alpha v\beta 3$-integrin epitopes on vasa vasorum in growing plaques results in marked inhibition of plaque angiogenesis in cholesterol fed rabbits. The unique mechanism of drug delivery for highly lipophilic agents such as paclitaxel contained within emulsions depends on close apposition between the nanoparticle carrier and the targeted cell membrane and has been described as "contact facilitated drug delivery." In contrast to liposomal drug delivery (generally requiring endocytosis), the mechanism of drug transport in this case involves lipid exchange or lipid mixing between the emulsion vesicle and the targeted cell membrane, which depends on the extent and frequency of contact between two lipidic surfaces. The rate of lipid exchange and drug delivery can be greatly increased by the application of clinically safe levels of ultrasound energy that increase the propensity for fusion or enhanced contact between the nanoparticles and the targeted cell membrane.

The combination of targeted drug delivery and molecular imaging with MRI has the potential to enable serial characterization of the molecular epitope expression based on imaging readouts. Monitoring and confirmation of therapeutic efficacy of the therapeutic agents at the targeted site would facilitate personalized medical regimens.

### *Antirestenosis Drugs Encapsulated in Biodegradable Nanoparticles*

Local delivery of antiproliferative drugs encapsulated in biodegradable nanoparticles has shown promise as an experimental strategy for preventing restenosis development. A novel PDGFR β-specific tyrphostin, AGL-2043 (Calbiochem), was formulated in polylactide-based nanoparticles and was administered intraluminally to the wall of balloon-injured rat carotid and stented pig coronary arteries (Banai et al. 2005). The antiproliferative effect of nanoencapsulated tyrphostin was found to be considerably higher than that of surface-adsorbed drug. In the pig model, intramural delivery of AGL-2043 resulted in reduced in-stent neointima formation in the coronary arteries as compared to control despite similar degrees of wall injury. The results of this study suggest that locally delivered tyrphostin AGL-2043 formulated in biodegradable nanoparticles may be applicable for antirestenotic therapy independent of stent design or type of injury.

### *Controlled Delivery of Nanoparticles to Injured Vasculature*

Optimal size of nanoparticles designed for systemic delivery is approximately 50–150 nm, but this size range confers a high surface area-to-volume ratio, which results in fast diffusive drug release. Spatial control has been achieved by biopanning a phage library to discover materials that target abundant vascular antigens exposed in disease (Chan et al. 2010). Temporal control is achieved by designing 60-nm hybrid nanoparticles with a lipid shell interface surrounding a polymer core, which is loaded with slow-eluting conjugates of paclitaxel for controlled ester hydrolysis and drug release over approximately 12 days. The nanoparticles inhibit human aortic smooth muscle cell proliferation in vitro and showed greater in vivo vascular retention during percutaneous angioplasty as compared to nontargeted controls. This nanoparticle technology may potentially be used toward the treatment of injured vasculature.

### *IGF-1 Delivery by Nanofibers to Improve Cell Therapy for Myocardial Infarction*

Strategies for cardiac repair include injection of cells, but these approaches have been hampered by poor cell engraftment, survival, and differentiation. To address these shortcomings for the purpose of improving cardiac function after injury, a self-assembling peptide nanofibers was designed for prolonged delivery of insulin-like growth factor 1 (IGF-1), a cardiomyocyte growth and differentiation factor, to the myocardium, using a “biotin sandwich” approach (Davis et al. 2006). Biotinylated IGF-1 was complexed with streptavidin and then bound to biotinylated

self-assembling peptides. This biotin sandwich strategy enabled binding of IGF-1 but did not prevent self-assembly of the peptides into nanofibers within the myocardium. IGF-1 that was bound to peptide nanofibers activated Akt, decreased activation of caspase-3, and increased expression of cardiac troponin I in cardiomyocytes. In studies on rats, cell therapy with IGF-1 delivery by biotinylated nanofibers improved systolic function after experimental myocardial infarction. This nanobiotechnology approach has the potential to improve the results of cell therapy for myocardial infarction, which is in clinical trials currently.

## *Injectable Peptide Nanofibers for Myocardial Ischemia*

Endothelial cells can protect cardiomyocytes from injury through platelet-derived growth factor (PDGF)-BB signaling. PDGF-BB induces cardiomyocyte Akt phosphorylation in a time-– and dose-dependent manner and prevents apoptosis via PI3K/Akt signaling. An experimental study in rats using injectable self-assembling peptide nanofibers, which bound PDGF-BB in vitro, demonstrated sustained delivery of PDGF-BB to the myocardium at the injected sites for 14 days (Hsieh et al. 2006). This blinded and randomized rat study showed that injecting nanofibers with PDGF-BB, but not nanofibers or PDGF-BB alone, decreased cardiomyocyte death and preserved systolic function after myocardial infarction. A separate blinded and randomized study showed that PDGF-BB delivered with nanofibers decreased infarct size after ischemia/reperfusion. PDGF-BB with nanofibers induced PDGFR-β and Akt phosphorylation in cardiomyocytes in vivo. These data demonstrate that PDGF-BB signaling and in vitro finding can be translated into an effective in vivo method of protecting myocardium after infarction. Furthermore, this study shows that injectable nanofibers allow precise and sustained delivery of proteins to the myocardium with potential therapeutic benefits.

## *Liposomal Nanodevices for Targeted Cardiovascular Drug Delivery*

High affinity ligand–receptor interactions have been exploited in the design and engineering of targeting systems that use a liposomal nanodevice for site-specific cardiovascular drug delivery. An example of application is atherothrombosis, a condition in which platelet activation/adhesion/aggregation is closely associated with vascular thrombotic events. Therefore, the majority of antithrombotic therapies have focused on drugs that impede platelet-activation pathways or block ligand-binding platelet integrins. In spite of reasonable clinical efficacy of these therapies, the magic bullet, a single drug and delivery system that selectively targets pathologically thrombotic environment without affecting hemostatic balance remains elusive. The use of anti-integrin/anticoagulant/anti-inflammatory drugs in conjunction

might be necessary to treat the multifactorial nature of pathological thrombogenesis. For this purpose, a nanoscale device that can carry such a combination selectively to a thrombotic site is being developed at the Department of Biomedical Engineering of Case Western Reserve University (Cleveland, OH). The liposomal nanodevice surface is modified by RGD (Arginine-Glycine-Aspartic Acid) motifs that specifically targets and binds activated platelets by virtue of the high affinity interaction between the RGD-motif and the integrin GPIIb-IIIa expressed on active platelets, potentially acting as a thrombus-targeted vector. The ability of such liposomes to compete with native ligand fibrinogen in specifically binding activated platelets has been accomplished using both in vitro and in vivo approaches. The results demonstrate feasibility of using liposomes as platelet-targeted devices for delivery of cardiovascular therapeutics. By utilizing a library of synthetic peptide/peptidomimetic ligands having binding affinity toward specific receptors expressed in cardiovascular biology, it is possible to manipulate the liposome surface-modification and hence dictate targeting specificity and affinity of the liposomal nanodevices.

## *Low Molecular Weight Heparin-Loaded Polymeric Nanoparticles*

Low molecular weight heparin (LMWH) nanoparticles are available as potential oral heparin carriers. The nanoparticles are formulated using an ultrasound probe by water-in-oil-in-water emulsification and solvent evaporation with polymers. The mean diameter of LMWH-loaded nanoparticles ranges from 240 to 490 nm and is dependent on the reduced viscosity of the polymeric organic solution. The highest encapsulation efficiencies are observed when Eudragit polymers are used in the composition of the polymeric matrix. The in vitro biological activity of released LMWH, determined by the anti-factor Xa activity with a chromogenic substrate, is preserved after the encapsulation process, making these nanoparticles good candidates for oral administration.

## *Nanoparticles for Cardiovascular Imaging and Targeted Drug Delivery*

The potential dual use of nanoparticles for both imaging and site-targeted delivery of therapeutic agents to cardiovascular disease offers great promise for individualizing therapeutics. Image-based therapeutics with site-selective agents should enable verification that the drug is reaching the intended target and a molecular effect is occurring. Experimental studies have shown that binding of paclitaxel to smooth muscle cells in culture has no effect in altering the growth characteristics of the cells. If paclitaxel-loaded nanoparticles are applied to the cells, however, specific binding elicits a substantial reduction in smooth muscle cell proliferation, indicating that selective targeting may be a requirement for effective drug delivery in this situation. Similar behavior has been demonstrated for doxorubicin-containing particles.

Intravenous delivery of fumagillin (an antiangiogenic agent)-loaded nanoparticles targeted to αvβ3-integrin epitopes on vasa vasorum in growing plaques results in marked inhibition of plaque angiogenesis in cholesterol fed rabbits. The unique mechanism of drug delivery for highly lipophilic agents such as paclitaxel contained within emulsions depends on close apposition between the nanoparticle carrier and the targeted cell membrane and has been described as "contact facilitated drug delivery." In contrast to liposomal drug delivery (generally requiring endocytosis), the mechanism of drug transport in this case involves lipid exchange or lipid mixing between the emulsion vesicle and the targeted cell membrane, which depends on the extent and frequency of contact between two lipidic surfaces. The rate of lipid exchange and drug delivery can be greatly increased by the application of clinically safe levels of ultrasound energy that increase the propensity for fusion or enhanced contact between the nanoparticles and the targeted cell membrane.

The combination of targeted drug delivery and molecular imaging with MRI has the potential to enable serial characterization of the molecular epitope expression based on imaging readouts. Monitoring and confirmation of therapeutic efficacy of the therapeutic agents at the targeted site would facilitate personalized medical regimens.

## *Nanofiber-Based Scaffolds with Drug-Release Properties*

Electrospinning is a versatile technique that enables the development of nanofiber-based scaffolds, from a variety of polymers that may have drug-release properties. Using nanofibers, it is now possible to produce biomimetic scaffolds that can mimic the extracellular matrix for tissue engineering (Ashammakhi et al. 2009). Nanofibers can guide cell growth along their direction. Combining factors like fiber diameter, alignment, and chemicals offers new ways to control tissue engineering. In vivo evaluation of nanomats included their degradation, tissue reactions, and engineering of specific tissues. New advances made in electrospinning, especially in drug delivery, support the massive potential of these nanobiomaterials. Nevertheless, there is already at least one product based on electrospun nanofibers with drug-release properties in a phase III clinical trial for wound dressing. Hopefully, clinical applications in tissue engineering will follow to enhance the success of regenerative therapies.

## *Nanotechnology Approach to the Vulnerable Plaque as Cause of Cardiac Arrest*

Recent studies have shown that plaque exists in two modes: non-vulnerable and vulnerable. The latter is the probable cause of death in sudden cardiac arrest. Blood passing through an artery exerts a shearing force and can cause vulnerable plaque to rupture, which often leads to occlusion and myocardial infarction. Approximately, 60–80% of sudden cardiac deaths can be attributed to the physical rupture of vulnerable plaque.

There is currently no satisfactory solution to the problem of vulnerable plaque but it will be tackled by a "Program of Excellence in Nanotechnology" by the National Heart, Lung, and Blood Institute of the NIH. In concert with the NIH's strategy to accelerate progress in medical research through innovative technology and interdisciplinary research, cardiac disease was chosen as the focus of the National Heart Lung and Blood Institute's Program of Excellence in Nanotechnology. The program will be a partnership of 25 scientists from The Burnham Institute (La Jolla, CA), University of California Santa Barbara, and The Scripps Research Institute (San Diego, CA) that will design nanotechnologies to detect, monitor, treat, and eliminate "vulnerable" plaques. By focusing on devising nanodevices, machines at the molecular level, the scientists at these institutions will specifically target vulnerable plaque. It is hoped that this work will lead to useful diagnostic and therapeutic strategies for those suffering from this form of cardiac disease. The project team will work on three innovative solutions to combat vulnerable plaque:

- Building delivery vehicles that can be used to transport drugs and nanodevices to sites of vulnerable plaque.
- Designing a series of self-assembling polymers that can be used as molecular nanostents to physically stabilize vulnerable plaque.
- Creating nanomachines comprised of human proteins linked to synthetic nanodevices for the purpose of sensing and responding to vulnerable plaque.

## Nanotechnology for Regeneration of the Cardiovascular System

Nanotechnology may facilitate repair and replacement of blood vessels, myocardium, and myocardial valves. It also may be used to stimulate regenerative processes such as therapeutic angiogenesis for ischemic heart disease. Cellular function is integrally related to morphology, so the ability to control cell shape in tissue engineering is essential to ensure proper cellular function in final products. Precisely constructed nanoscaffolds and microscaffolds are needed to guide tissue repair and replacement in blood vessels and organs. Nanofiber meshes may enable vascular grafts with superior mechanical properties to avoid patency problems common in synthetic grafts, particularly small-diameter grafts. Cytokines, growth factors, and angiogenic factors can be encapsulated in biodegradable microparticles or nanoparticles and embedded in tissue scaffolds and substrates to enhance tissue regeneration. Scaffolds capable of mimicking cellular matrices should be able to stimulate the growth of new heart tissue and direct revascularization.

Nanostructures promote formation of blood vessels, bolster cardiovascular function after heart attack. Scientists at the Institute of Bionanotechnology in Medicine at Northwestern University (Evanston, Ill) have shown that injecting nanoparticles into the hearts of mice that suffered heart attacks helped restore cardiovascular function in these animals. The finding is an important research advance that one day could help rapidly restore cardiovascular function in people who have heart disease.

The self-assembling nanoparticles – made from naturally occurring polysaccharides and molecules known as peptide amphiphiles – boost chemical signals to nearby cells that induce formation of new blood vessels and this may be the mechanism through which they restore cardiovascular function. One month following injection, the hearts of the treated mice were capable of contracting and pumping blood almost as well as healthy mice. In contrast, the hearts of untreated mice contracted about 50% less than normal.

Cellular function is integrally related to morphology, so the ability to control cell shape in tissue engineering is essential to ensure proper cellular function in final products. Precisely constructed nanoscaffolds and microscaffolds are needed to guide tissue repair and replacement in blood vessels and organs. Nanofiber meshes may enable vascular grafts with superior mechanical properties to avoid patency problems common in synthetic grafts, particularly small-diameter grafts. Cytokines, growth factors, and angiogenic factors can be encapsulated in biodegradable microparticles or nanoparticles and embedded in tissue scaffolds and substrates to enhance tissue regeneration. Scaffolds capable of mimicking cellular matrices should be able to stimulate the growth of new heart tissue and direct revascularization.

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# Chapter 7
# Cell Therapy for Cardiovascular Disorders

## Introduction

New cell-based therapeutic strategies are being developed in response to the shortcomings of available treatments for heart disease. Potential repair by cell grafting or mobilizing endogenous cells holds particular attraction in heart disease, where the meager capacity for cardiomyocyte proliferation likely contributes to the irreversibility of heart failure. Cell therapy approaches include attempts to reinitiate cardiomyocyte proliferation in the adult, conversion of fibroblasts to contractile myocytes, conversion of bone marrow (BM) stem cells into cardiomyocytes, and transplantation of myocytes or other cells into injured myocardium. Basics and techniques of cell therapy have been described in a special report on this topic (Jain 2011). Applications in cardiovascular disorders will be described in this chapter including methods of delivery of cells.

## Types of Cell Therapy for Cardiovascular Disorders

New cell-based therapeutic strategies are being developed in response to the shortcomings of available treatments. Potential repair by cell grafting or mobilizing endogenous cells holds particular attraction in heart disease, where the meager capacity for cardiomyocyte proliferation likely contributes to the irreversibility of heart failure. Cell therapy approaches include attempts to reinitiate angiogenesis, cardiomyocyte proliferation in the adult, conversion of fibroblasts to contractile myocytes, conversion of bone marrow stem cells into cardiomyocytes, and transplantation of myocytes or other cells into injured myocardium. Cells may be incorporated in tissue engineering for cardiac diseases. Tepha Inc's technology allows surgeons to use a patient's own cells in combination with a scaffold of absorbable biomaterial, which can be implanted and not only function properly, but also guide the regeneration of new valvular tissue. Most of this chapter will deal with the use of cells in the treatment of cardiovascular diseases. However, there are other approaches which have effect on cardiovascular health mediated through cells.

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**Table 7.1** Classification of various types of cell therapy for cardiovascular disorders

| |
|---|
| *Immune modulation of inflammation by cytokines released from cells* |
| Role of intrinsic circulating cells |
| *Cells derived from extra-cardiovascular tissues* |
| Splenic myocytes for repair of the injured heart |
| Cells derived from the skeletal muscle: myoblasts, myoendothelial cells |
| *Cells derived from the heart* |
| Cardiac progenitor cells |
| Cardiomyocytes |
| *Stem cells* |
| Autologous adult stem cells: HSCs from blood for bone marrow, MSCs from bone marrow |
| Cardiac stem cells |
| Cord blood stem cells |
| Embryonic stem cells converted into cardiomyocytes |
| *Implantation of genetically engineered cells* |
| Transplantation of cells secreting vascular endothelial growth factor |
| Transplantation of genetically modified bone marrow stem cells |
| *Induction of cellular proliferation and regeneration* |
| Drug-induced proliferation of cardiomyocytes |
| Drug-induced proliferation of stem cells |
| *Cells for cardiac tissue repair/engineering* |
| Cells for reconstruction of blood vessels and valves of the heart |
| Fibroblast culture for patching damaged heart |
| Fetal cardiomyocytes for seeding into cardiac grafts |



An example of this is immune modulation. A classification of various types of cell therapy for cardiovascular disorders is shown in Table 7.1.

## *Cell-Mediated Immune Modulation for Chronic Heart Disease*

Inflammation is a normal response of the immune system to cellular injury caused by infection, trauma, or other stimuli. During the inflammatory process, immune cells release a number of factors, including cytokines that modulate inflammation and facilitate the healing process. While this inflammatory process is usually self-limiting, it can persist, become chronic, and lead to a number of serious medical conditions including chronic heart disease.

Oxidative stress is a factor known to initiate apoptosis, a physiologic process that is inherently anti-inflammatory. Oxidative stress can induce cell apoptosis and during this process signaling molecules, including phosphatidylserine (PS), normally present on the inner surface of the cell membrane, become exposed on the cell surface. The PS molecules interact with specific PS receptors on the surface of antigen presenting cells (APCs) of the immune system, including macrophages and

dendritic cells (DCs). The interaction with macrophages leads to an upregulation in the production of the anti-inflammatory cytokines IL-10 and TGF-β. DCs that interact with apoptotic cells remain immature and, in the presence of anti-inflammatory cytokines such as IL-10 and TGF- β, cause the differentiation of some naive T cells to regulatory T cells. These traffic through the tissues and inhibit inflammatory cells such as T1 cells by a process that includes cell–cell interaction and the production of anti-inflammatory cytokines by the regulatory T cells. The end result is a reduction in tissue levels of inflammatory cytokines such as TNF-α, IL-6, IFN-γ, and IL-1 β, and a downregulation of chronic inflammation.

Celacade™ technology (Vasogen Inc) targets the inflammation underlying CHF. During a brief outpatient procedure, a small sample of a patient's blood is drawn into a disposable cartridge and exposed to controlled oxidative stress utilizing a proprietary technology. The treated blood is then administered to the same patient intramuscularly. An initial course of treatment comprising three consecutive outpatient procedures is administered over a 2-week period, and treatments are continued once per month thereafter. Celacade™ is in phase III clinical development.

## *Human Cardiovascular Progenitor Cells*

A basic understanding of the development of the human heart is essential for devising cell-based therapies for heart disease. The functional heart is comprised of distinct mesoderm-derived lineages including cardiomyocytes, endothelial cells, and vascular smooth muscle cells. Studies in the mouse embryo and the mouse ESC differentiation model have provided evidence indicating that these three lineages develop from a common Kdr cardiovascular progenitor that represents one of the earliest stages in mesoderm specification to the cardiovascular lineages. To determine whether a comparable progenitor is present during human cardiogenesis, a study has analyzed the development of the cardiovascular lineages in hESC differentiation cultures (Yang et al. 2008). After induction with combinations of activin A, BMP4, FGF2, VEGF, and dickkopf homolog 1 (DKK1) in serum-free media, hESC-derived embryoid bodies generate a KDRlow/C-KITneg population that displays cardiac, endothelial, and vascular smooth muscle potential in vitro and, after transplantation, in vivo. When plated in monolayer cultures, these KDRlow/C-KITneg cells differentiate to generate populations consisting of greater than 50% contracting cardiomyocytes. Populations derived from the KDRlow/C-KITneg fraction give rise to colonies that contain all three lineages when plated in methylcellulose cultures. Results of dilution studies and cell-mixing experiments support the interpretation that these colonies are clones, indicating that they develop from a cardiovascular colony-forming cell. Together, these findings identify a human cardiovascular progenitor that defines one of the earliest stages of human cardiac development.

BM-derived circulating endothelial progenitor cells (EPCs) have been reported to play a role in postnatal vasculogenesis through vessel regeneration and remodeling. These cells have been reported to mobilize into the blood stream in response to vascular injury, and differentiate into cells expressing a host of endothelial cell

markers in vitro. Because of demonstrable regenerative capacity in animal models of human disease, EPCs are considered to represent a novel treatment option for problematic cardiovascular disorders such as myocardial infarction (MI) and peripheral vascular disease (PVD). Various studies have been performed to test the clinical efficacy of EPCs in patients with cardiovascular disease (CVD), including the mobilization of EPCs with pharmacologic agents in patients with heart disease, and harvesting of cells from the circulation and BM for autologous reinfusion in affected patients. The outcomes of these trials have been mixed and not as robust as predicted from the animal models, partly because of the variation in the definition of human EPCs and the resulting heterogeneity in cell populations used in treatments. In studies that have been conducted to examine cell therapies for treatment of CVD, efficacy of treatment with putative EPCs has been inconsistent, and some aspects of these trials may need to be redesigned for future successful treatment of CVD (Mund et al. 2009).

Benefits in clinical trials of treatment of heart disease with BM and skeletal muscle progenitors had marginal positive results perhaps because adult stem cells have limited plasticity. An early stage of cardiovascular progenitors, characterized by expression of OCT4, stage-specific embryonic antigen 1 (SSEA-1) and mesoderm posterior 1 (MESP1), has been derived from human pluripotent stem cells treated with the cardiogenic morphogen BMP2 (Blin et al. 2010). This progenitor population was multipotential and able to generate cardiomyocytes as well as smooth muscle and endothelial cells. When transplanted into the infarcted myocardium of immunosuppressed nonhuman primates, an SSEA-1+ progenitor population derived from Rhesus ESCs differentiated into ventricular myocytes and reconstituted 20% of the scar tissue. Primates transplanted with an unpurified population of cardiac-committed cells, which included SSEA-1– cells, developed teratomas in the scar tissue, whereas those transplanted with purified SSEA-1+ cells did not. The authors believe that the SSEA-1+ progenitors described by them have the potential to be used in cardiac regenerative medicine.

## *Inducing the Proliferation of Cardiomyocytes*

There has been a longstanding controversy as to whether cardiomyocytes (cardiac muscle cells) in adult mammals have the capacity to proliferate. It was generally believed that once the heart has fully developed, cardiomyocytes lose the molecular plasticity that allows them to divide. Consequently, acutely injured mammalian hearts do not regenerate but scar. One important mechanism used by mammalian cardiomyocytes to control cell cycle is p38 MAP kinase activity. A new study demonstrates that adult cardiomyocytes lacking p38 activity continue to proliferate through many rounds of cell division in the presence of fibroblast growth factor-1 (Engel et al. 2005). Genetic studies of p38 inhibition have shown that it regulates genes thought to be critical for cardiomyocyte proliferation. These findings represent

the first step toward showing that drugs that eliminate p38 activity could reduce scar tissue formation and enhance cardiac regeneration after cardiac injury. Clinical application of the approach is still far off because the mechanism of the effect observed may not be the only one and other possible mechanisms may regulate cardiomyocyte proliferation.

## *Role of the SDF-1-CXCR4 Axis in Stem Cell Therapies for Myocardial Ischemia*

During development, the SDF1-CXCR4 axis plays a critical role in gradient-guided cell movements, and in adults it is involved in retention and mobilization of stem cells. Since SDF-1 is upregulated during hypoxic tissue damage, strategies to augment or stabilize SDF-1 have been utilized to target blood-derived stem cells to ischemic tissue. This concept was exploited by preventing SDF1 degradation with dipeptidylpeptidaseIV (DPPIV) inhibition and mobilization of stem cells by G-CSF after acute myocardial infarction (Zaruba and Franz 2010). This targeted CD34+ CXCR4+ cells to ischemic heart and attenuated ischemic cardiomyopathy. Thus preserving functional SDF1 by DPPIV inhibition after ischemia may enhance stem cell therapies.

## *Role of Splenic Myocytes in Repair of the Injured Heart*

Monocytes circulate freely and patrol blood vessels but differentiate irreversibly into DCs or macrophages upon tissue entry. Undifferentiated monocytes reside in the spleen and outnumber their equivalents in circulation. The reservoir monocytes assemble in clusters in the cords of the subcapsular red pulp and are distinct from macrophages and DCs. In response to ischemic myocardial injury, splenic monocytes increase their motility, exit the spleen en masse, accumulate in injured tissue, and participate in wound healing (Swirski et al. 2009). These observations uncover a role for the spleen as a site for storage and rapid deployment of monocytes for cardiac repair. A novel 3D optical imaging technique (fluorescence molecular tomography), developed at the Massachusetts General Hospital's Center for Molecular Imaging Research (Boston, MA) enabled study of monocyte-mediated immune functions at the site of heart muscle injury. This method revealed that the monocytes that travel to the heart after a heart attack come directly from the spleen and that, without the splenic monocytes, the heart tissue does not heal well. The investigators also found that the hormone angiotensin II, known to be released in response to a heart attack, is actively involved in the release of monocytes from the spleen. Identifying that pathway could lead to ways of manipulating the splenic monocyte reservoir to improve healing after a heart attack.

## *Reprogramming of Fibroblasts into Functional Cardiomyocytes*

The reprogramming of fibroblasts to induced pluripotent stem cells (iPSCs) raises the possibility that a somatic cell could be reprogrammed to an alternative differentiated fate without first becoming a stem/progenitor cell. A large pool of fibroblasts exists in the postnatal heart, yet no single "master regulator" of direct cardiac reprogramming has been identified. A combination of three developmental transcription factors (Gata4, Mef2c, and Tbx5) have been shown to rapidly and efficiently reprogram postnatal cardiac or dermal fibroblasts directly into differentiated cardiomyocyte-like cells (Ieda et al. 2010). Such induced cardiomyocytes express cardiac-specific biomarkers, have a global gene expression profile similar to cardiomyocytes, and contract spontaneously. Fibroblasts transplanted into mouse hearts 1 day after transduction of the three factors also differentiated into cardiomyocyte-like cells. These findings demonstrate that functional cardiomyocytes can be directly reprogrammed from differentiated somatic cells by defined factors. Reprogramming of endogenous or transplanted fibroblasts might provide a source of cardiomyocytes for regenerative approaches to the damaged heart. Much work remains to be done before these findings can be translated into clinical use. Some of the issues that need to be resolved are (Murry and Pu 2011):

- The efficiency of generating functioning cardiomyocytes requires improvement as only ~1% of reprogrammed myocytes appeared to be bona fide beating cardiomyocytes.
- Reprogramming systems should be developed without use of integrating viruses.
- There is need to determine if the induced cardiomyocyte state persists over the long term. The investigators should demonstrate that these cells can integrate into the injured heart, beat synchronously with the host myocardium, and restore electrical and contractile function.

## *Small Molecules to Enhance Myocardial Repair by Stem Cells*

The clinical success of stem cell therapy for myocardial repair hinges on a better understanding of cardiac fate mechanisms. Small molecules involved in cardiac fate have been identified by screening a chemical library for activators of the signature gene Nkx2.5, using a luciferase knockin bacterial artificial chromosome in mouse P19CL6 pluripotent stem cells (Sadek et al. 2008). A family of sulfonylhydrazone (Shz) small molecules, particularly, Shz-3 has been shown to trigger cardiac mRNA and protein expression in a variety of embryonic and adult stem/progenitor cells, including human mobilized peripheral blood mononuclear cells (M-PBMCs) when cultured for 3 days, and then for 7 days without the drug. Small-molecule-enhanced M-PBMCs engrafted into the rat heart in proximity to an experimental injury improved cardiac function better than control cells. Recovery of

cardiac function correlated with persistence of viable human cells, expressing human-specific cardiac mRNAs and proteins. Thus, Shz small molecules are promising starting points for drugs to promote myocardial repair/regeneration by activating cardiac differentiation in M-PBMCs. Shz compounds do not appear to be toxic in mice, and because the human M-PBSCs are washed for 7 days after treatment, the compounds are likely not to be harmful to humans. Further studies will examine precisely what the Shz drugs are doing to the cells, and to identify additional chemical signals that might drive the cells toward a more mature form of heart cell.

## Cell Therapy for Atherosclerotic Coronary Artery Disease

The injured endothelial monolayer of coronary arteries is regenerated by circulating bone marrow-derived EPCs, which accelerates re-endothelialization and limits atherosclerotic lesion formation. However, risk factors for coronary artery disease such as age and diabetes reduce the number and functional activity of these circulating endothelial progenitor cells, thus limiting the regenerative capacity. The impairment of stem/progenitor cells by risk factors may contribute to atherogenesis and atherosclerotic disease progression.

Intensive investigation is underway to determine the functional activity of EPCs for endothelial repair and vascular protection. Preliminary data suggest that EPC have the capacity to regenerate the injured endothelial monolayer and thereby reduce atherosclerotic lesion formation. Transplantable bone marrow-derived endothelial progenitor cells may represent novel therapeutic targets for vascular disease. Improvement in EPC levels and/or function by pharmacological interventions could therefore be an attractive novel therapeutic option for antiatherosclerotic therapy. In addition to replacing injured endothelial cells in the lining of blood vessels, EPC also promote new vessel formation. It is still controversial whether plaque angiogenesis accelerates atherosclerotic lesion development. Further studies are necessary to elucidate the definitive contribution of circulating progenitor cells for prevention of atherosclerosis.

### *MyoCell™ (Bioheart)*

MyoCell™ (Bioheart) is the tissue regeneration therapy product for the treatment of a continuum of cardiovascular disease states ranging from heart attack to end-stage heart disease. Autologous myoblasts are isolated from skeletal muscle biopsy material. The MyoCell™ therapy consists of a proprietary transport and injection media, biopsy enzyme disassociation process, myoblast selection process, culture media, and subsequent transplant into the damaged heart muscle. The enzyme dissociation process selects a very specific subpopulation of myoblasts, and the injection media keeps cells quiescent for up to 8 days before fusion that allows the muscle to build up gradually instead of suddenly, which minimizes reaction and

promotes better muscle formation. The Bioheart MyoCell™ process ensures that the active ingredient consists of a cell population with the propensity to engraft, proliferate, adapt to the cardiac microenvironment, and support cardiac workload. The myoblasts have been delivered via percutaneous catheter system as well as part of a coronary artery bypass graft surgery.

In 2001, the first percutaneous nonsurgical transplantation was performed by injecting cultured autologous myoblasts from a biopsy of the patient's thigh muscle (Bioheart Inc's MyoCell) to a damaged area of a patient's heart following myocardial infarction. The procedure was performed by a team of interventional cardiologists at the Thorax Center of the University of Rotterdam in the Netherlands. Ten injections totaling 25 million cells were made into the damaged portion of the patient's heart using an endoventricular catheter introduced into the patient's groin. The patient was discharged within 24 h and was doing well at home 3 days later. This study was part of a safety evaluation of the Bioheart's MyoCell product that has now progressed to phase III clinical trials in Europe. This may be one of the most significant developments in the history of treating heart disease patients.

## *Cardiac Stem Cells*

The average heart contains approximately 600 million myocytes. They are lost at a rate of approximately 100 million myocytes per year. However, the heart of an 80-year-old person still has approximately 400 million myocytes. It is assumed that there is a mechanism for regeneration, despite the old belief about the heart being a postmitotic organ with lack of regeneration of myocytes. Mitosis was identified in myocytes more than 50 years ago. Cardiac side population cells are a distinct cardiac progenitor cell population, capable of cardiomyogenic differentiation into mature cardiomyocytes through a process mediated by cellular coupling with adult cardiomyocytes. According to the new paradigm, parenchymal and nonparenchymal cells are continuously replaced by newly formed younger populations of myocytes as well as by vascular smooth muscle and endothelial cells (Anversa et al. 2006). Heart homeostasis is regulated by a stem cell compartment characterized by multipotent cardiac stem cells that possess the ability to acquire the distinct cell lineages of the myocardium.

The number of stem cells and progenitor cells identified in normal myocardium increases in transplanted hearts. When the heart is transplanted it suffers some damage but the recipient's stem cells pitch in to help heal the new organ by migrating to it. The Y chromosome can be used to detect migrated undifferentiated cells expressing stem-cell antigens and to discriminate between primitive cells derived from the recipient and those derived from the donor.

In 2006, three different teams announced discovery of so-called master heart cells that hold the promise of treating patients with serious cardiovascular disease. Each team identified cardiovascular "precursor" cells from cultures of mouse ESCs. It is very likely that these versatile cells will also be found in the embryonic

human heart, raising hopes of 1 day repairing and "rejuvenating" damaged hearts by growing these embryonic stem cell (ESC) lines in a laboratory. Two of the groups, one at the Massachusetts General Hospital in Boston, the other at Mount Sinai School of Medicine in New York, claimed that their precursors give rise to three types of cells in the heart. Cardiac muscle cells can be grown, as well as the smooth muscle in the walls of the blood vessels that supply the heart and crucial endothelial cells that line the coronary blood vessels. The third team at the Children's Hospital in Boston identified precursors for cardiac and smooth muscle.

There is evidence that a persistent population of immature progenitor cells is present in the mature myocardium. These cell populations probably represent stages along a continuum of cardiac stem cell (CSC) development and differentiation. Uncommitted cardiac progenitor cells(CPCs) have been isolated from cardiac ventricle, which appear to resemble the more immature, common pool of embryonic lateral plate mesoderm progenitors that yield both myocardial and endocardial cells during normal cardiac development (Ott et al. 2007). Under controlled in vitro conditions and in vivo, these cells can differentiate into endothelial, smooth muscle, and cardiomyocyte lineages and can be isolated and expanded to clinically relevant numbers from adult rat myocardial tissue.

## *Cardiomyocytes Derived from Epicardium*

A previously unknown cardiac myocyte lineage, which derives from the proepicardial organ, has been identified in mouse. These progenitor cells, which express the T-box transcription factor Tbx18, migrate onto the outer cardiac surface to form the epicardium, an epithelial sheet overlying the heart, and then make a substantial contribution to myocytes in the ventricular septum and the atrial and ventricular walls (Cai et al. 2008). Tbx18-expressing cardiac progenitors also give rise to cardiac fibroblasts and coronary smooth muscle cells. Another study has reported novel cardiogenic precursor marked by expression of the transcription factor Wt1 and located within the epicardium (Zhou et al. 2008). During normal murine heart development, a subset of these Wt1+ precursors differentiated into fully functional cardiomyocytes. The pluripotency of these epicardial progenitor cells form the basis of future efforts for cardiac regeneration and repair. This progenitor population contains all the potential to regenerate multiple tissue types within the heart and not just the cardiomyocytes. The investigators now want to know whether the epicardium in an adult mouse could be induced to make cardiomyocytes, which would be much more translatable to human studies. Other ongoing questions are whether this newly discovered progenitor is truly multipotent, how multipotency is controlled, and whether this can be used therapeutically to benefit adults with heart failure. The fact that the Wt1-expressing progenitors can also differentiate into fibroblasts in the developing heart suggests that they can contribute to scar formation in the adult heart after injury. Efforts will be made to turn the progenitors away from making scars, and steer them toward making cardiomyocytes.

## Methods of Delivery of Cells to the Heart

Various cell delivery methods have been investigated for cell transplantation treatment of cardiac infarcts. Cells may be injected directly into the area of the myocardial infarct or various devices such as catheters may be used to deliver cells into the coronary circulation. A rat model of ischemic cardiomyopathy was used to compare the efficacy of intramyocardial and intracoronary angiogenic cell precursors (ACP) implantation (Sun et al. 2008). Human ACPs, delivered via intramyocardial or intracoronary injection, engrafted into damaged cardiac tissue and improved cardiac function within 4 weeks through effects on scar morphology and blood vessel formation. Significant reductions in myocardial scar area were only observed in the intramyocardial ACP group, although increases in blood vessel density were greater in the intracoronary ACP group than in the intramyocardial delivery group.

### *Cellular Cardiomyoplasty*

A very promising approach to reversal or stabilization of the postinfarct remodeling process is the direct injection of regenerative cells into the myocardial infarct scar. Such cell-based therapy for cardiac repair is called cellular cardiomyoplasty. This procedure can be conducted with MyoCath™, Bioheart's proprietary catheter delivery system, to facilitate MyoCell™ delivery into the myocardium via the retrograde catheterization of the left ventricular cavity. Because implantation of autologous skeletal myoblasts has been shown to lead to replacement of nonfunctioning myocardial scar with functioning muscle and improvement in myocardial performance, myoblast implantation at the time of coronary artery bypass graft or via the endoventricular approach may lead to the same effects. In principle, myoblast implantation by catheter delivery may offer the same therapeutic benefit. MYOHEART (Myogenesis Heart Efficiency and Regeneration Trial) is a phase I, open-label, nonrandomized, dose escalation, multicenter study that is in progress to assess the safety and cardiovascular effects of MyoCell™ implantation by MyoCath™ in congestive heart failure (CHF) following myocardial infarction with previous placement of an implantable cardioverter defibrillator.

### *IGF-1 Delivery by Nanofibers to Improve Cell Therapy for MI*

Strategies for cardiac repair by injection of cells have been hampered by poor cell engraftment, survival, and differentiation. To address these shortcomings for the purpose of improving cardiac function after MI, a self-assembling peptide nanofibers was designed for prolonged delivery of insulin-like growth factor 1 (IGF-1), a cardiomyocyte growth and differentiation factor, to the myocardium, using a "biotin sandwich" approach (Davis et al. 2006). Biotinylated IGF-1 was complexed with

streptavidin and then bound to biotinylated self-assembling peptides. This biotin sandwich strategy enabled binding of IGF-1 but did not prevent self-assembly of the peptides into nanofibers within the myocardium. IGF-1 that was bound to peptide nanofibers activated Akt, decreased activation of caspase-3, and increased expression of cardiac troponin I in cardiomyocytes. In studies on rats, cell therapy with IGF-1 delivery by biotinylated nanofibers improved systolic function after experimental MI, demonstrating how engineering the local cellular microenvironment can improve cell therapy.

### *Noninvasive Delivery of Cells to the Heart by Morph®guide Catheter*

Most methods of delivery of cells to the myocardium are invasive. Morph®guide catheter (Biocardia) is a FDA-approved noninvasive device, which is inserted like a standard angioplasty catheter through a blood vessel in the groin, but instead of advancing to the coronary artery, the catheter is advanced into the ventricle of the heart so that it can deliver directly into the heart muscle using a small helical needle. Twenty patients have been treated to date with this system delivering bone marrow-derived cells through BioCardia's Investigational Helical Infusion System with excellent safety results and an average total procedure time from insertion to removal of catheters of only 43 min. This method of delivery is currently in use at the University of Miami in a clinical trial on heart failure patients comparing two cell populations: autologous adult bone marrow derived mononuclear cells and adult autologous MSCs. These cell populations have excellent safety profiles in clinical studies performed to date. As cells are taken from the patient's own bone marrow, there will be no issues of rejection of the cells as foreign by the patient's immune system.

## Cell Therapy for Cardiac Revascularization

### *Transplantation of Cardiac Progenitor Cells for Revascularization of Myocardium*

Cell transplantation for revascularization has been explored as an alternative to bypass surgery for severe coronary atherosclerosis. According to one report, c-kit-positive CPCs activated with IGF-1 and HGF before their injection in proximity of the site of occlusion of the left coronary artery in rats, engrafted within the host myocardium forming temporary niches (Tillmanns et al. 2008). Subsequently, CPCs divided and differentiated into endothelial cells and smooth muscle cells and, to a lesser extent, into cardiomyocytes. The acquisition of vascular lineages appeared to be mediated by the upregulation of HIF 1α, which promoted the synthesis and

secretion of stromal-derived factor 1 (SDF-1) from hypoxic coronary vessels. SDF-1 was critical in the conversion of CPCs to the vascular fate. CPCs formed conductive and intermediate-sized coronary arteries together with resistance arterioles and capillaries. The new vessels were connected with the primary coronary circulation, and this increase in vascularization more than doubled myocardial blood flow in the infarcted myocardium. This beneficial effect, together with myocardial regeneration, attenuated postinfarction dilated myopathy, reduced infarct size, and improved function. In conclusion, locally delivered activated CPCs generate de novo coronary vasculature and may be implemented clinically for restoration of blood supply to the ischemic myocardium.

## *Stem Cells to Prevent Restenosis After Coronary Angioplasty*

Percutaneous transluminal coronary angioplasty is used to widen the stenosis of the atherosclerotic coronary arteries without an open surgical procedure. It is carried out by positioning a catheter with a small inflatable balloon on the end within the narrowed portion of the artery. The balloon is then inflated, which dilates the stenosed segment of the artery by compressing the atherosclerotic plaque and stretching the wall of the artery. This procedure is usually followed by insertion of a stent at the site of stenosis to prevent restenosis. Restenosis — the reclosure or narrowing of an artery — can be a concern with coronary stents and with balloon angioplasty procedures. Restenosis occurs when blockages return a few weeks or months after the coronary stent procedure. Bare metal stents are now being replaced by drug-eluting stents (DES) but there are still complications such as thrombosis of the stent.

Scientists at Sheffield University (Yorkshire, UK) are developing the world's first regenerative stent utilizing stem cells. The body reacts to the presence of the stent as a foreign body. By using the patient's own stem cells to line the stent, one can prevent the reaction. Supportive evidence comes from another study, which showed that stent coating with an integrin-binding cyclic Arg-Gly-Asp peptide may be useful for reducing in-stent restenosis by accelerating endothelialization through recruitment of endothelial progenitor cells (Blindt et al. 2006).

Axordia, in collaboration with Lombard Medical Technologies PLC, is developing a new generation of treatment for coronary artery disease – a regenerative stent. The collaboration between Lombard and Axordia is for a two and a half year project to develop a regenerative stent that encourages rather than restricts local vascular repair. Attaching Axordia's proprietary, stem cell-derived endovascular cells to Lombard Medical's PEP™ programmable polymer coating on the stent surface shall allow the human body to promote controlled vascular repair and heal the damaged coronary artery vessel wall.

The stent shall represent a major step forward into a new format for stent technology where early healing (endothelialization) is promoted by stem cells rather than using cytotoxic drugs on drug eluting stents (DES), which inhibit growth and can cause late stage thrombotic events. As a result, this stent technology could

revolutionize the DES market. A caveat for the use of stem cells for vascular repair is that these cells may be the culprits in the development of restenosis. Scientists at the Weill Cornell Medical College in New York are currently studying how stem cells implant themselves in the wall of arteries and grow out of control following angioplasty and contribute to development of re-stenosis. The researchers observed that transforming growth factor beta (TGF-β), which stimulates tissue growth, is released in high levels inside the artery following the trauma of angioplasty. This could happen because TGF-β may attract stem cells to the injured area to heal the wound, leading to the growth of dense tissue, which blocks the artery. If this mechanism of restenosis is proven, one strategy would be to incorporate a TGF-β antagonist in the DES to shut off this response.

## Role of Cells in Cardiac Tissue Repair

### *Modulation of Cardiac Macrophages for Repair of Infarct*

A new strategy has been investigated for the modulation of cardiac macrophages to a reparative state at a predetermined time after MI with the aim to promote resolution of inflammation and elicit infarct repair (Harel-Adar et al. 2011). Intravenous injections of PS-presenting liposomes are employed to mimic the anti-inflammatory effects of apoptotic cells. Following PS-liposome uptake by macrophages in vitro and in vivo, the cells secrete high levels of anti-inflammatory cytokines (TGF-β and IL-10) with upregulation of the expression of the mannose receptor CD206, concomitant with downregulation of proinflammatory biomarkers, such as TNF-α and the surface marker CD86. In a rat model of acute MI, targeting of PS-presenting liposomes to infarct macrophages after injection via the femoral vein was demonstrated by MRI. The treatment promotes angiogenesis, preserves small scars, and prevents ventricular dilatation and remodeling. This strategy represents a unique and accessible approach for MI repair.

### *Transplantation of Myoblasts for Myocardial Infarction*

Cells taken from peripheral muscles and injected into infarcted areas of the myocardium are incorporated into the myocardium and take on some characteristics of cardiomyocytes. Most importantly, the cell transplants improve contractility in the infarcted areas. To prevent the progression of events after experimental MI, autologous skeletal myoblasts (muscle precursor cells) have been transplanted into infarcted myocardium of the rabbits induced by a cryoprobe. Follow-up histological examination of the heart showed that islands of different sizes comprising elongated, striated cells that retained characteristics of both skeletal and cardiac cells were found in the area of the infarction. In rabbits in which myoblasts were

incorporated, myocardial performance was improved. The ability to regenerate functioning muscle after autologous myoblast transplantation could have an important effect on patients after acute MI.

After MI, adverse remodeling with left ventricular dilatation is a major determinant of poor outcome. Skeletal myoblast implantation improves cardiac function post-MI by modulation of adverse remodeling, and this effect can be significantly enhanced by targeting IL-1 as a key upstream regulator of both adverse remodeling and graft cell death. Several studies have shown that implanted skeletal myoblasts form viable grafts in infarcted myocardium, resulting in enhanced post-MI exercise capacity and contractile function with attenuated ventricular dilation. These data illustrate that syngeneic myoblast implantation after MI improves both in vivo and ex vivo indexes of global ventricular dysfunction and deleterious remodeling and suggests that cellular implantation may be beneficial after MI. Skeletal autologous myoblasts have been the first cells to be used clinically in patients with advanced ischemic heart failure as they feature several advantages. Preliminary data from a phase I clinical trial suggested the feasibility and safety of autologous skeletal myoblast transplantation in severe ischemic cardiomyopathy.

The Myoblast Autologous Graft in Ischemic Cardiomyopathy (MAGIC) trial was designed to test whether autologous skeletal myoblasts can be used to reverse damage done to cardiac muscle following a heart attack, or to safely halt a patient's further progression of heart failure. Patients who participated in the study had ischemic heart failure. A previous blockage in one of their coronary arteries caused a heart attack, creating a serious defect in the wall of their left ventricle (LV). Patients were also diagnosed with an additional blockage in a coronary artery requiring coronary artery bypass grafting (CABG) surgery. In the study, one of two doses of autologous skeletal myoblasts or placebo were injected during CABG surgery in and around the scar tissue caused by the heart attack to attempt to strengthen the defect in the ventricular wall. Patients were enrolled at sites in France, UK, Italy, Belgium, and Germany. All patients received an implantable cardioverter-defibrillator. The primary efficacy end points were the 6-month changes in global and regional LV function assessed by echocardiography. The safety end points comprised a composite index of major cardiac adverse events and ventricular arrhythmias. It was concluded that myoblast injections combined with CABG in patients with depressed LV function failed to improve echocardiographic heart function (Menasché et al. 2008). The increased number of early postoperative arrhythmic events after myoblast transplantation, as well as the capability of high-dose injections to revert LV remodeling, warrants further investigation.

## *Patching Myocardial Infarction with Fibroblast Culture*

Revascularization of damaged myocardium represents an important therapeutic goal in patients with coronary artery disease. Pharmacologically induced revascularization with VEGF and bFGF has been successful in stimulating new microvessel growth or an increase in myocardial collateral blood flow.

A scaffold-based, three-dimensional, human dermal fibroblast culture (3DFC) has been tested as a cardiac patch to stimulate revascularization and preserve left ventricular function following infarction in severe combined immunodeficient (SCID) mice (Kellar et al. 2005). The 3DFC contains viable cells that secrete angiogenic growth factors and has been previously shown to stimulate angiogenesis. These results show that the 3DFC as a cardiac patch functioned to attenuate further loss of left ventricular function accompanying acute myocardial infarct, and that this may be related in part to myocardial revascularization.

## *Cardiac Repair with Myoendothelial Cells from Skeletal Muscle*

A novel population of myoendothelial cells have recently been identified and purified from human skeletal muscle. These cells coexpress myogenic and endothelial cell markers and produce robust muscle regeneration when injected into cardiotoxin-injured skeletal muscle (Okada et al. 2008). Myoendothelial cells stimulated the growth of new blood vessels in the heart and were more effective at repairing the injured cardiac muscle and reducing scar tissue than previous approaches that have used muscle myoblasts. At 6 weeks after injection, the myoendothelial cell-injected hearts functioned 40–50% more effectively compared with hearts that had been injected with myoblasts, thereby dramatically improving the function of the injured left ventricle. These cells represent a novel cell population from human skeletal muscle that may hold promise for cardiac repair as an autologous transplant. This means that for a patient who suffers a heart attack, myoendothelial cells can be isolated from a muscle biopsy, purified, and then reinjected into the injured heart muscle, thereby avoiding any risk of rejection by introducing foreign cells.

## *Myocardial Tissue Engineering*

Myocardial tissue engineering involves seeding cells in 3D matrices of biodegradable polymers or cell sheet engineering without artificial scaffolds to form new myocardial constructs. Functional assembly of engineered cardiac muscle can be enhanced by oxygen supply provided by mechanisms resembling those in normal vascularized tissues. To mimic the capillary network, cardiomyocytes and fibroblasts isolated from the neonatal rat hearts were cultured on a highly porous elastomer with a parallel array of channels that were perfused with culture medium (Radisic et al. 2006). To mimic oxygen supply by hemoglobin, culture medium was supplemented with a perfluorocarbon (PFC) emulsion; constructs perfused with unsupplemented culture medium served as controls. Consistently, constructs cultivated in the presence of PFC contained higher amounts of DNA and cardiac biomarkers (troponin I, connexin-43) and had significantly better contractile properties as compared to control constructs. In both groups, electron microscopy revealed open channels and the presence of cells at the channel surfaces as well as within

constructs. Improved properties of cardiac constructs could be correlated with the enhanced supply of oxygen to the cells, by a combined use of channeled scaffolds and PFC.

A type I collagen–glycosaminoglycan (GAG) scaffold cross-linked by dehydrothermal (DHT) treatment has been investigated for the implantation of adult bone marrow-derived MSCs into the infarcted region in the rat heart (Xiang et al. 2006). Most of the DHT cross-linked collagen–GAG scaffolds degraded. A substantial amount of neovascularization was seen in the infarcted region in the implant groups and in the scaffolds themselves. BrdU-positive cells appeared both in the degraded scaffold and the infarct region. DHT cross-linked collagen–GAG scaffolds warrant continued investigation as delivery vehicles for implantation of cells into infarcted cardiac tissue.

A new method has been described to engineer 3D contractile bioengineered heart muscle (BEHM) using primary cardiac myocytes isolated from hearts of neonatal rats and a biodegradable fibrin gel (Huang et al. 2007). Over the course of several days, the fibrin breaks down after fulfilling its role as a temporary support for the cells. Comparison of two different ways of using fibrin gel as a basis for creating BEHM: layering on top of the gel, and embedding within it, showed that the former produced a more cohesive tissue that contracted with greater force. This method has several advantages over current approaches to cardiac tissue bioengineering. Measurement showed that the BEHM that had formed in just 4 days after a million cells were layered on fibrin gel could contract with an active force of more than 800 μN. Although it is still only about half the force generated within the tissue of an actual beating heart, it is much higher than the forces created by engineered heart tissue samples grown and reported by other researchers. Fibrin hydrogel yields a product that is ready within a few days, that spontaneously organizes and begins to contract with a significant and measurable force, and that responds appropriately to external factors such as calcium. It brings scientists another step closer to the goal of creating replacement parts for damaged human hearts, or eventually growing an entirely new heart from a small number of cardiac myocytes. BEHM is now being transplanted into the hearts of rats that have suffered heart attacks to see if the new tissue can repair the damage. Further studies will use bioreactors that will expose the BEHM tissue to more of the nutrients and other conditions that are present in the body. Although BEHM is still years away from use as a human heart failure treatment, or as a testing ground for new cardiovascular drugs, the method would help accelerate progress toward those goals.

Researchers at the Massachusetts Institute of Technology have developed a novel 3D scaffold that could be seeded with living heart cells or stem cells that could be developed into a patch of cardiac tissue for treating congenital heart defects, or to aid the recovery of tissue damaged by a heart attack (Engelmayr et al. 2008). The biodegradable scaffold is gradually absorbed into the body, leaving behind new tissue. The accordion-like honeycomb is the first to be explicitly designed to match the structural and mechanical properties of native heart tissue. As a result, it has several advantages over previous cardiac tissue engineering scaffolds.

# Role of Stem Cells in Repair of the Heart

## *Role of Stem Cells in Cardiac Regeneration Following Injury*

The ability to regenerate damaged heart tissue is present in all vertebrate species but, for unknown reasons, humans and other mammals have "turned off" this regeneration function. Although multiple types of progenitor (undifferentiated stem) cells have been identified within the mammalian heart, it displays little or no regeneration when damaged. In contrast, when a portion of a zebrafish's heart is removed, it regenerates and activation of Notch signaling pathway precedes the regeneration. A new study shows that regeneration proceeds through two coordinated stages following resection of the ventricular apex of the zebrafish heart: (1) a blastema is formed, comprising of progenitor cells that express precardiac markers, undergo differentiation, and proliferate; and (2) tissue surrounding both cardiac chambers induces developmental markers and rapidly expands, creating a new epithelial cover for the exposed myocardium (Lepilina et al. 2006). A subpopulation of these epicardial cells undergoes epithelial-to-mesenchymal transition, invades the wound, and provides new vasculature to regenerating muscle. During regeneration, the ligand fgf17b is induced in myocardium, while receptors fgfr2 and fgfr4 are induced in adjacent epicardial-derived cells. When FGF signaling is experimentally blocked by expression of a dominant-negative Fgf receptor, neovascularization fails, prematurely arresting regeneration. These findings reveal injury responses by myocardial and epicardial tissues that collaborate in an FGF-dependent manner to achieve cardiac regeneration. Future studies in zebrafish could help us discover why this regenerative ability is lacking in mammals and potential ways to stimulate it. Discovering the key to this dormant ability could lead to new ways to treat human hearts damaged by disease.

## *Cardiomyocytes Derived from Adult Skin Cells*

In 2010, Prof. Robert Schwartz of the University of Houston, Texas, devised a method for converting ordinary human skin cells into heart cells. Although adult skin cells have been converted into stem cells previously, this method requires fewer steps. The cells developed are similar to ESCs and ultimately can be made into early stage heart cells derived from a patient's own skin. These then could be implanted and grown into fully developed functioning heart cells, reversing the damage caused by previous heart attacks. These new cells would replace the damaged cardiac tissue that weakens the heart's ability to pump, develops into scar tissue, and causes arrhythmias. Early clinical trials using these reprogrammed cells on actual heart patients are planned. It may be possible to grow an entirely new heart or other organ from these reprogrammed cells.

## *Cardiomyocytes Derived from ESCs*

Resources for cardiomyocytes to repair the heart are still limited and ESCs are a potential source. The derivation of the hESC lines offers a number of potential advantages over the currently available candidate donor cells (Caspi and Gepstein 2006).

- hESC can provide an unlimited number of human cardiac cells for transplantation.
- The ability of ESC to differentiate into a multiple cell lineages may be used for transplantation of different cell types such as endothelial progenitor cells for induction of angiogenesis or specialized pacemaker cells.
- ESC-derived cardiomyocytes could lend themselves to extensive characterization and genetic manipulation to promote desirable characteristics such as resistance to ischemia and apoptosis or improved contractile function.
- ESC-derived cells could be used as a vehicle for gene therapy to manipulate local myocardial environment, for example, by secretion of growth-promoting factors.

The ability to generate potentially unlimited numbers of cardiomyocytes ex vivo from the hESC may also bring a unique value to tissue engineering approaches. Role of cardiomyocytes derived from ESCs is shown in Fig. 7.1.

## *Studies to Identify Subsets of Progenitor Cells Suitable for Cardiac Repair*

All heart cells are not equal in responsiveness to signals to differentiate; some respond, whereas others do not. To investigate the development of the heart, investigators have focused on CASTOR (CST), a gene that has been implicated in stem cell differentiation in the fruit fly, but a study was based on manipulated the gene in frogs because heart development has been mostly learned in frogs (Christine and Conlon 2008). Oligonucleotides were used to mask the portions of genetic material that calls for the assembly of the CST protein in frogs and heart development was observed in the frog embryos. Instead of all the cells differentiating in harmony, a small subset of cells at the base of the heart remained in a state of infancy. These progenitor cells normally would have given rise to the outer walls of ventricles of the adult heart. This finding has implications for the use of progenitor cells for therapy, in which cells would be transplanted into the area of the organ injured by a heart attack in order to create healthy tissue. This study contradicts the current concept that all progenitors are the same. Instead, there appears to be at least two or more types of progenitors. The discovery, made in frogs may lead to advances in understanding and ultimately treating congenital heart disease and heart attacks.

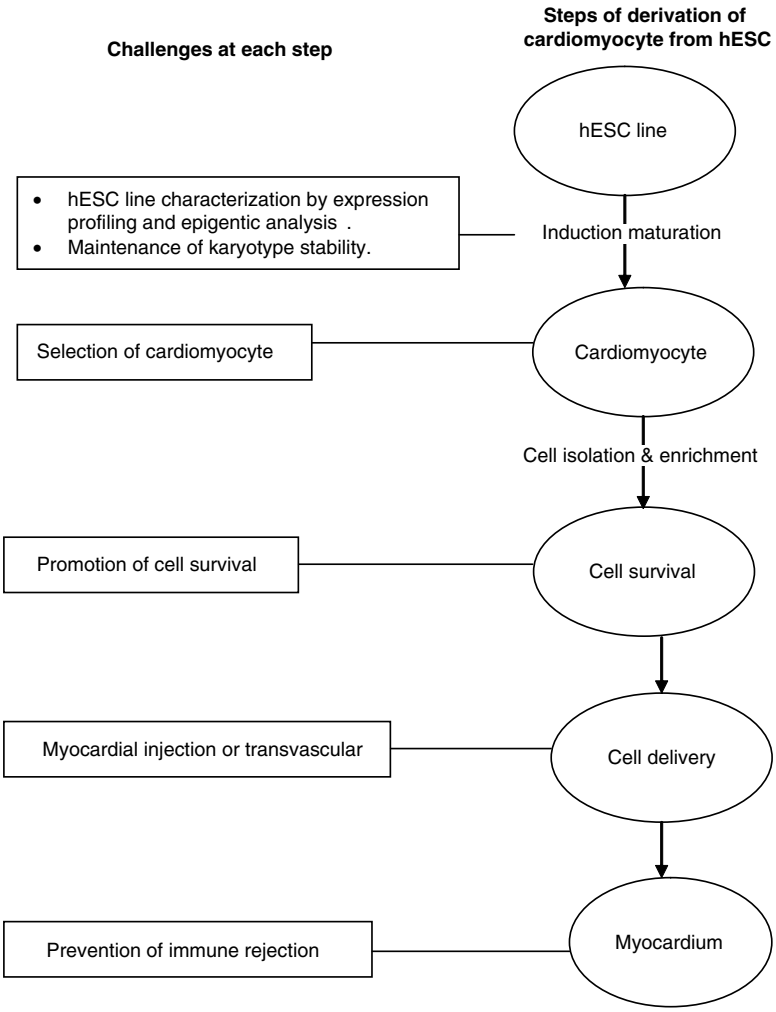


**Fig. 7.1** hESC-derived cardiomyocytes from laboratory to bedside (© Jain PharmaBiotech)

Not only does it explain how stem cells differentiate to create the heart, but this knowledge may be useful for designing strategies to repair heart muscle after a heart attack. However, in order to identify the subsets of cells that would be the most appropriate for treating a heart attack, one must figure out just how many different types of heart cell progenitors exist in the first place. Efforts are being made to determine this by manipulating other genes in addition to CST. These findings may be verified in an intermediary organism, such as a mouse model before searching for genetic counterparts in humans.

## *Technologies for Preparation of Stem Cells for Cardiovascular Therapy*

### Pravastatin for Expansion of Endogenous Progenitor and Stem Cells

The mechanisms of the favorable effects of statins on the heart have largely focused on the blood vessel wall. Stabilization of atherosclerotic plaques and improvement in endothelial-mediated blood vessel relaxation are considered to be the major explanations for their beneficial actions. Pravastatin, one of the statins currently prescribed to lower cholesterol, can also improve cardiac function after myocardial infarction through an anti-inflammatory mechanism, rather than by induction of therapeutic angiogenesis. One study has demonstrated that pravastatin promotes differentiation from mononuclear cells to endothelial progenitor cells, but has an inhibitory effect on muscle progenitor cells (Kusuyama et al. 2006). These findings suggest a previously unreported mechanism of the effect of statin therapy on vascular progenitor cells. Another study supports anti-inflammatory mechanism and no synergistic effect for inducing angiogenesis was found by the combination of pravastatin and implantation of bone marrow mononuclear cells (Li et al. 2006).

Earlier reports have shown that HMG-CoA reductase inhibitors, known as statins, increased the number of circulating bone marrow-derived or hematopoietic stem cells (HSCs) in blood but most work has focused on their effects in improving blood flow. Pravastatin is now known to increase the concentration of endogenous stem cells that may participate in cardiac repair independent of any cholesterol-lowering action. High doses of pravastatin improve cardiac function and coronary blood flow in animal models in which flow has been artificially restricted, creating a condition known as hibernating myocardium. In this condition, heart cells reduce their function and oxygen needs and become dormant in response to insufficient blood flow.

### Cytokine Preconditioning of Human Fetal Liver CD133+ SCs

Poor results of previous studies, where donor stem cells were injected directly into the damaged heart tissue, suggested that something was missing – either the conditions in the surrounding tissue were not suitable or a more pluripotent stem cell was needed that could readily co-differentiate into both muscle and blood vessels in order to initiate rapid revascularization of the regenerating heart tissue. The discovery that a specific type of human fetal stem cell can co-differentiate simultaneously into both muscle and blood vessel cells may unlock the door to therapies that replace damaged tissue in the heart and other organs. Stem cells bearing a surface antigen called CD133 are capable of differentiating into both angiogenic and myogenic cells (Shmelkov et al. 2005). While cells bearing this CD133 biomarker are very rare in adult tissues, they are particularly abundant in the fetal liver. CD133+ fetal liver cells ultimately may be used for therapeutic angiomyogenesis. However, areas of cardiac damage often lack essential biological cues critical for stem-cell

differentiation and preconditioning of stem cells in the laboratory may be required before transplanting them to the site of injury within the heart. Preconditioning of these CD133+ cells with cytokines vascular endothelial growth factor-A (VEGF-A) and brain-derived nerve growth factor (BDNF) enhances the angiomyogenesis. One drawback is the possible rejection of fetal stem cell transplant. To explore the possibility of autologous stem cell therapy, researchers are investigating to find if human adult livers might bear traces of CD133+ stem cells or stimulation of bone marrow stem cells with these same growth factors might also generate myocytes and angiogenic cells.

### Expansion of Adult Cardiac Stem Cells for Transplantation

In human and animal studies, scientists at Johns Hopkins (Baltimore, MD) have developed a fast and safe method for collecting cardiac stem cells (CSCs) from small amounts of biopsied heart tissue obtained through cardiac catheterization in patients undergoing treatment for heart failure. CSCs are expanded in the laboratory to produce enough cells within 4 weeks that can be used to repair heart tissue clinically. The resulting clusters, called cardiospheres, contain cells that retain the ability to regenerate themselves, and to develop into more specialized heart cells that can conduct electrical currents and contract like the heart muscle should. These findings, if confirmed in further clinical trials, could offer patients a means of using their own SCCs to repair heart tissue soon after they have suffered a myocardial infarction, or to regenerate weakened muscle resulting from heart failure, perhaps averting the need for heart transplants. Use of a person's own adult CSCs instead of those from a donor avoids the risk of triggering an immune response that could cause rejection.

In rats subjected coronary occlusion, intravascular injection of CSCs after reperfusion limits infarct size, and ameliorates left ventricular function (Dawn et al. 2005). This study demonstrated that CSCs are effective when delivered in a clinically relevant manner, a clear prerequisite for clinical application, and that these beneficial effects are independent of cell fusion. The results establish CSCs as candidates for cardiac regeneration and support an approach in which the heart's own stem cells could be collected, expanded, and stored for subsequent therapeutic repair.

### Role of MSCs in Growth of CSCs

Mesenchymal stromal cells (MSC) are present in the adult mouse heart. They can be isolated from the mouse heart and promote growth and differentiation of adult CSC (Lushaj et al. 2010). These findings could have a significant beneficial impact on future heart failure treatment. Coculture and co-implantation of cardiac-derived MSC with adult CSC could provide extensive cardiac regeneration and maintenance of the CSC population after implantation into the heart.

## *Role of ESCs in Repair of the Heart*

Investigators have been looking for a relatively simple way to help restore the functioning of hearts damaged by chemotherapy, which can occur in up to 10% of cancer patients treated with chemotherapy drugs. ESCs are known to repair damaged hearts. In mice, hESCs use different methods to morph into two kinds of cells needed to restore heart function: cardiac muscle cells that contract the heart as well as the endothelial cells that line blood vessels found throughout the organ. In one study, 2 months after mice with ailing hearts were treated with hESCs, about 2% of cells in their heart showed evidence of a human genetic marker (Zhang et al. 2004). hESCs primarily fuse onto mouse cardiac cells to produce new muscle cells that have both human and mouse DNA. But to form new blood vessel cells, they differentiate by themselves, presumably to patch damaged mouse blood vessels with human cells. These findings should help resolve debate within the field as to whether ESC transfer actually creates new types of cells that last within a heart.

ESCs represent a source for cell-based regenerative therapies of heart failure. The pluripotency and the plasticity of ESCs allow them to be committed to a cardiac lineage following treatment with growth factors of the transforming growth factor (TGF)-β superfamily. French researchers have designed a protocol to turn on expression of cardiac-specific genes in undifferentiated murine ESCs stimulated with BMP2 and/or TGF-β (Zeineddine et al. 2005). Cell commitment results in a significant improvement in spontaneous cardiac differentiation of ESCs both in vitro and in vivo. In further studies, these researchers have successfully transplanted ESCs from mice to repair myocardial infarction in sheep (Menard et al. 2005). Sheep in ESC-treated group had healthier heart tissue after 1 month compared with sheep who did not receive ESC transplantation (the control group). There was no rejection or tumor formation following the procedure. This shows that mouse ESCs could be transplanted into larger mammals to regenerate damaged heart cells, strengthening the possibility that ESCs could one day be used to repair heart cells in humans.

Studies in animal models have shown that transplanted mouse ESCs can regenerate infarcted myocardium in part by becoming cardiomyocytes, vascular smooth muscle, and endothelial cells that result in an improvement in cardiac structure and function (Singla et al. 2006). Therefore, ESCs hold promise for myocardial cellular therapy. One important finding of this study is that the implanted cells did not result in tumor formation, which is one of the primary safety concerns for stem cell therapy. That undifferentiated hESCs can survive in rat hearts during myocardial infarction without the formation of teratoma, has been demonstrated by using undifferentiated GFP-transgenic hESCs (Xie et al. 2007). A laser-capture microscope was used to dissect the GFP-positive cell area from the hESC-injected hearts to document the expression of human cardiac-specific genes including GATA-4, Nkx-2.5, and cardiac troponin I. However, like cancer cells, ESCs have a capacity to reproduce indefinitely, and scientists must perfect cell transplant methods that are safe before the therapy can be attempted in human patients. Future studies will explore ways to refine the cell types used in treating heart disease to enhance safety.

There is a need for new heart valves, which it is estimated will be needed for 80,000 persons worldwide by the year 2020. Currently valves used for replacements are obtained either from pigs or made artificially from metal, but they are not as efficient or durable as human valves and wear out after 10–15 years. Heart muscles could also be grown from stem cells in the laboratory, and would function like the patient's own heart tissue. Adipose-derived stem cells (ADCs), which are capable of differentiating into cells with phenotypic and functional features of endothelial cells, are being investigated as a source of interstitial cells to populate tissue-engineered heart valve constructs (Colazzo et al. 2010).

### ESC Transplantation for Tumor-Free Repair of the Heart

The propensity of ESCs for multilineage differentiation carries the liability of neoplastic growth, impeding therapeutic application. In one approach, the tumorigenic threat associated with ESC transplantation was suppressed by cardiac-restricted transgenic expression of the reprogramming cytokine TNF-α, enhancing the cardiogenic competence of recipient heart (Behfar et al. 2007). The in vivo aptitude of TNF-α to promote cardiac differentiation was recapitulated in embryoid bodies in vitro. The procardiogenic action required an intact endoderm and was mediated by secreted cardioinductive signals. Researchers discovered approximately 15 proteins whose production was dramatically increased after TNF-α stimulation. These proteins, when combined into a cocktail, secured guided differentiation of ESCs, producing cardiac progenitors called cardiopoietic cells, which are cardiac specified cell precursors rather than any cell type. Characterized by a downregulation of oncogenic markers, upregulation, and nuclear translocation of cardiac transcription factors, this predetermined population yielded functional cardiomyocyte progeny. Such guided heart precursor cells did not form tumors, even though they were transplanted at doses that would otherwise carry a high risk for tumorigenesis with ESCs. Recruited cardiopoietic cells delivered in infarcted hearts generated cardiomyocytes that proliferated into scar tissue, integrating with host myocardium for tumor-free repair. Thus, cardiopoietic programming establishes a strategy to hone stem cell pluripotency, offering a tumor-resistant approach for regeneration of the heart after myocardial infarction.

## *Transplantation of Stem Cells for Acute Myocardial Infarction*

Several stem cell technologies have been used for treatment of MI. Several of these are in clinical trials.

### Autologous Bone Marrow-Derived Stem Cell Therapeutics

The improvement in cardiac function after migration of autologous bone marrow-derived stem cell (BMSCs) to the heart can be explained by their paracrine effects, inducing angiogenesis and preventing ischemic myocardium from apoptosis (Brunner et al. 2008). These effects may explain why the number of circulating

BMSCs is directly correlated with cardiovascular risk and life expectancy. Exercise and hormones are physiological stimuli for the mobilization of BMSCs, whereas cardiovascular risk factors severely reduce their number and functions. Current cardiovascular medications increase the amounts of autologous BMSCs.

AMR001 (Amorcyte Inc) consists of autologous BMSCs isolated from adult bone marrow using a cytokine gradient. The isolation gradient used by Amorcyte identifies cells that respond to cytokine signals released as a response to myocardial infarction. The cells based on this biologic activity sets AMR001 apart from other autologous treatments in development in two ways; (1) the homogeneity of the cell population in AMR001 should provide more consistent efficacy than a heterogeneous mixture of bone marrow-derived cells; (2) cells' response to infarction-specific cytokine signals enables them to penetrate the peri-infarct zone upon injection into the infarct-related artery. This is important because often a major limitation in cell therapy is being able to target and penetrate the damaged area to deliver therapeutics.

The process of harvesting, purifying, and re-administering the cells to the patient is minimally invasive and can be completed within 24–48 h. Both the harvesting and the re-administration are performed in a conscious patient under sedation. AMR001 completed phase I clinical trials in 2009 demonstrating feasibility, safety, and biologic activity at a threshold dose. This is the first stem cell trial in AMI ever conducted that has prospectively established a significant relationship between dose and effect. If successful, AMR001 use will be expanded into chronic ischemia and congestive heart failure.

### Autologous Bone Marrow-Derived Mesenchymal Precursor Stem Cells

Texas Heart Institute is treating heart attack patients with autologous bone marrow-derived mesenchymal precursor stem cells (MPCs) to promote better healing and to prevent congestive heart failure. A randomized phase I trial has enrolled 25 patients in three phases in which the patient receives 25, 75, or 150 million MPCs, which are injected into damaged but still viable areas of the heart muscle. Preclinical trials have established that 10 days after the heart attack is the optimal time to give this treatment. The heart is still inflamed in the days just after a heart attack and if one waits too late to administer MPCs, the heart will develop much scar tissue and its ability to pump will already be compromised. 3D imaging technology is used to map the electrical and mechanical function of the left ventricle. The study is sponsored by Angioblast Systems, a company which provides the proprietary MPCs. MPCs can also be administered via a catheter.

### Transplantation of Cord Blood Stem Cells

Cord blood-derived stem cells (CBSCs) have been transplanted into the acutely ischemic lateral wall of the left ventricle (LV) in a porcine model of acute MI (Ghodsizad et al. 2009). Transplantation of CBSCs significantly improved LV function and prevented scar formation as well as LV dilation. Since differentiation, apoptosis, and macrophage mobilization at infarct site were excluded as underlying

mechanisms, paracrine effects are most likely to account for the observed effects of CBSC treatment. It is possible that CBSCs may release nourishing substances that rally primitive cells within the heart itself to form new blood vessels and muscle.

### Transplantation of hESCs

hESCs have been cultured and the hESC-derived cardiomyocytes display structural and functional properties of early stage cardiomyocytes. This unique differentiation system may have significant impact on the study of early human cardiac differentiation, functional genomics, pharmacological testing, cell therapy, and tissue engineering. It seems likely that if placed in an adult human heart, these cells would produce heart muscle cells.

Geron Corporation's scientists and collaborators have demonstrated that hESC-derived cardiomyocytes, when injected directly into myocardial infarction zone in rats, improve cardiac structure and contractile function as shown by echocardiography and MRI (LaFlamme et al. 2007). This is the first study to document the potential clinical utility of regenerating damaged heart muscle by injecting hESC-derived cardiomyocytes directly into the site of the infarct. In addition, the research confirms the effectiveness of a scalable production system that enables Geron to manufacture the cardiomyocytes for use in ongoing large animal studies and, ultimately, testing in humans. To enable survival in the heart, the hESC-derived cardiomyocytes were suspended in a cocktail of survival factors that had been experimentally determined to dramatically enhance cell survival after injection into the infarcted ventricular wall. Four weeks later, tissue sections from the infarcted hearts were examined for the presence of the human cells. The vast majority of human cardiomyocytes were localized in the central region of the infarct, suggesting that the cells were capable of engraftment in the hostile environment of the infarct zone. Moreover, a portion of the cardiomyocytes was mitotic after injection, possibly enhancing their regenerative efficiency. The grafts also induced a brisk, host-derived angiogenic response: all the implants contained numerous capillaries lined with rat endothelial cells.

### Transplantation of HSCs

Stem cells can sense injury to a distant organ and migrate to the site of damage to undergo cell differentiation to promote structural and functional repair. This high degree of stem cell plasticity led to studies to test whether dead myocardium following infarction in mice could be restored by transplanting bone marrow cells (BMCs). Results of these studies indicate that locally delivered bone marrow cells can generate de novo myocardium, ameliorating the outcome of coronary artery disease.

Highly enriched HSCs, the so-called side population (SP) cells, were transplanted into lethally irradiated mice subsequently rendered ischemic by coronary artery occlusion for 60 min followed by reperfusion (Jackson et al. 2001). The engrafted SP or their progeny migrated into ischemic cardiac muscle and blood

vessels, differentiated to cardiomyocytes and endothelial cells, and contributed to the formation of functional tissue. These results demonstrate the cardiomyogenic potential of HSCs and suggest a therapeutic strategy that eventually could benefit patients with myocardial infarction.

In one study, autologous bone marrow stem cells were injected into the infarct border zone in six patients who had had a myocardial infarction and had undergone CABG (Stamm et al. 2003). All patients were alive and well 3–9 months after surgery. Global left ventricular function was enhanced in four patients, and infarct tissue perfusion had improved strikingly in five patients. The authors believe that implantation of stem cells into the heart is safe and might induce angiogenesis, thus improving perfusion of the infarcted myocardium. Another study reported that the hematopoietic stem cells (HSCs) are unable to replace heart muscle after a heart attack, which refuted earlier findings (Balsam et al. 2004). The authors found that in mice, HSCs lodge in damaged hearts but retain the form of blood cells rather than transforming into muscle cells. This study differs from previous studies in that the investigators took whole bone marrow from mice and then isolated several purified groups of cells, including a highly purified subset of stem cells that can go on to form all blood cell types. Previous experiments had only used less purified cells. However, this study does not rule out the potential of stem cells in repairing the heart. Transplanted blood-forming cells may recruit new blood vessels to the damaged tissues. These new blood vessels may keep heart muscle cells alive that would otherwise have died, thus indirectly rescuing the heart. By genetically engineering those cells to make additional factors to recruit blood vessels, they may become part of a successful therapy.

### Transplantation of Autologous Angiogenic Cell Precursors

Vescell™ (TheraVitae) contains autologous angiogenic cell precursors (APCs) that are isolated from the patient's blood and expanded in the laboratory prior to transplantation in patients with myocardial infarction. They are administered by either infusion into the coronary arteries or injected directly into the myocardium. A clinical trial is in progress for treatment of angina due to myocardial ischemia where other therapies have failed. APCs are injected via a catheter into the coronary arteries for this trial. Only patients where an unoccluded coronary artery is available for infusion of cells are eligible for this trial.

### Transplantation of Adipose-Derived Stem Cells

Of all the adult stem cells potentially available for cell therapy, human adipose tissue-derived cells exhibit the greatest potential because of their capacity for differentiation. They possess a large number of practical advantages. Ironically, patients who suffer heart attacks are often obese, which is one of the risk factors. Autologous stem cells can be obtained in abundance by lipoaspiration from these patients.

A study has demonstrated that ADCs will engraft in injured heart muscle following a heart attack-like injury (Strem et al. 2005). The study evaluated three groups

of 12 mice: one group received a heart attack-like injury and ADCs (experimental), a second group received a heart attack-like injury and saline injection in place of ADCs (negative control), and a third group received ADCs but no heart attack-like injury (sham injury). Each ADC-treated mouse received 1 million cells. Each group was divided equally into three subgroups whose hearts were evaluated at 1, 7, and 14 days, respectively, following induction of heart injury. ADCs were detected in the damaged areas of the injured hearts of the experimental group at 1, 7, and 14 days following injury and were not detected in the negative control and sham injured groups. The ADCs in the injured areas of the experimental group also expressed cell markers consistent with cardiac muscle cells at day 14, suggesting the cells differentiated into cardiac muscle. Higher levels of ADCs were observed at the later time points, suggesting the ADCs may be proliferating or that they continue to circulate and engraft into the damaged area over time. Additionally, it was observed that the areas immediately surrounding the infarct regions express markers of endothelial cells, suggesting the ADCs may participate in inducing blood vessel growth or repair.

A clinical trial that started in Spain, designated as the "PRECISE" study, primarily evaluated safety and feasibility of use of adipose-derived stem cells in 36 patients with chronic myocardial ischemia. Cytori's Celution stem and regenerative cell processing system extracted and concentrated a patient's cells in the catheterization suite so they are available for the physician to reinject into damaged heart muscle in about an hour. A variety of clinical functional and imaging endpoints have been assessed in the study. The outcomes of the study will be evaluated after a follow-up.

Future challenges for the use of adult stem cells derived from adipose tissue will be to characterize several different stem cell types that exist in this tissue and to determine their differentiation potential. Advances in cryopreservation in the future may allow us to preserve cells isolated by lipoaspiration at 40 years of age, in order to treat a defective organ at 60 or 70 years of age (Roche et al. 2007).

### Intracoronary Infusion of Bone Marrow-Derived Cells for AMI

Bone marrow from adult humans contains endothelial precursors with phenotypic and functional characteristics of embryonic hemangioblasts, and these can be used to directly induce new blood vessel formation in the infarct bed (vasculogenesis) and proliferation of preexisting vasculature (angiogenesis) after experimental myocardial infarction. The neoangiogenesis results in decreased apoptosis of hypertrophied myocytes in the peri-infarct region, long-term salvage and survival of viable myocardium, reduction in collagen deposition, and sustained improvement in cardiac function. The use of cytokine-mobilized autologous human bone marrow-derived angioblasts for revascularization of infarcted myocardium (alone or in conjunction with currently used therapies) has the potential to significantly reduce morbidity and mortality associated with left ventricular remodeling. Advanced Cell Technology scientists have described a method for generating large numbers of hemangioblasts, which are newly characterized stem cells capable of differentiating

into both hematopoietic and angiogenic cells. These cells reduced the mortality rate after myocardial infarction and restored blood flow in hind limb ischemia in mouse models (Lu et al. 2007).

The Transplantation of Progenitor Cells And Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) trial investigates both safety, feasibility, and potential effects on parameters of myocardial function of intracoronary infusion of either circulating progenitor cells (CPC) or bone marrow-derived progenitor cells (BMC) in patients with AMI (Schachinger et al. 2006). Results of this pilot trial from Goethe University (Frankfurt, Germany) show that intracoronary infusion of progenitor cells (either BMC or CPC) is safe and feasible in patients after acute myocardial infarction, successfully revascularized by stent implantation. Both the excellent safety profile and the observed favorable effects on left ventricular remodeling provide the rationale for larger randomized double-blind trials. According to release of the results of this study in 2005, heart attack survivors whose hearts were infused with HSCs from their own bone marrow showed nearly twice the improvement in the organ's pumping ability as patients given a placebo. A further analysis of the data revealed that benefits to heart function seen 4 months after an attack appeared to be most pronounced in patients with more severe heart attacks that caused greater damage to the muscle. The primary goal of the 204-patient study was to show improvement in function of the left ventricle, which is considered a good indicator of a patient's prognosis following a heart attack. Both groups in the study had nearly identical left ventricular function at the start, and both showed improvement after 4 months as expected. However, patients who received the bone marrow cell infusion showed 5.5% improvement in their left ventricular ejection fraction compared to 3% improvement in the placebo group. The results are much better than those seen in previous smaller studies of cardiac cell therapy. There was less cardiac enlargement and improved blood flow to the infarcted region indicating the possibility that new blood vessels may have formed to nourish the damaged area. The reduction in cardiac enlargement seen in the HSC transplant patients appeared to lead to reduced incidence of new heart attacks, hospitalization due to heart failure, and deaths.

### Intracoronary Infusion of Mobilized Peripheral Blood Stem Cells

Mobilization of peripheral blood stem cells (PBSCs) with granulocyte colony stimulating factor (G-CSF) and intracoronary infusion has been studied as an alternative to invasive bone marrow cell collection. In a randomized trial in South Korea of patients with MI who had undergone coronary stenting, G-CSF therapy with intracoronary infusion of PBSC showed improved cardiac function, and promoted angiogenesis (Kang et al. 2004). However, aggravation of restenosis was a serious problem and further enrolment in the study was stopped. A possible explanation of this adverse effect of differentiation of G-CSF-mobilized progenitor cells into smooth muscle cells within the stented segment. The authors cautioned that in future studies with G-CSF-based stem-cell therapy, patients should be carefully monitored for unexpected effects.

### Transplantation of Endothelial Cells

Endothelial cells, either freshly isolated from embryonic vessels or established as homogenous cells in culture, differentiate into beating cardiomyocytes and express cardiac markers when cocultured with neonatal rat cardiomyocytes or when injected into postischemic adult mouse heart. Human umbilical vein endothelial cells also differentiate into cardiomyocytes under similar experimental conditions and transiently coexpress von Willebrand factor and sarcomeric myosin. In contrast, neural stem cells, which efficiently differentiate into skeletal muscle, differentiate into cardiomyocytes at a low rate. Fibroblast growth factor 2 and bone morphogenetic protein 4, which activate cardiac differentiation in embryonic cells, do not activate cardiogenesis in endothelial cells or stimulate transdifferentiation in coculture, suggesting that different signaling molecules are responsible for cardiac induction during embryogenesis and in successive periods of development. The fact that endothelial cells can generate cardiomyocytes sheds additional light on the plasticity of endothelial cells during development and opens perspectives for cell autologous replacement therapies in myocardial infarction.

### Transplantation of Cardiomyocytes Differentiated from hESCs

Cardiomyocytes differentiated from hESCs have been shown to engraft when transplanted into the rat heart (Laflamme et al. 2005). Both host and graft-derived angiogenesis (new blood vessel formation) was observed, critical to sustaining the viability of the graft. No evidence of tumor formation was seen. The results provide proof of concept that transplanted hESC-derived cardiomyocytes survive and retain properties of cardiomyocytes, important for their use in the treatment of heart failure and myocardial infarction. Geron corporation is currently transplanting hESC-derived cardiomyocytes in acute and chronic infarct animal models.

Scientists at Axiogenesis have investigated and examined the fate and functional impact of bone marrow (BM) cells and ESC-derived cardiomyocytes after transplantation into the infarcted mouse heart (Kolossov et al. 2006). This proved particularly challenging for the ESCs, as their enrichment into cardiomyocytes and their long-term engraftment and tumorigenicity are still poorly understood. They generated transgenic ESCs expressing puromycin resistance and enhanced green fluorescent protein cassettes under control of a cardiac-specific promoter. Puromycin selection resulted in a highly purified cardiomyocyte population, and the yield of cardiomyocytes increased six- to tenfold because of induction of proliferation on purification. Long-term engraftment (4–5 months) was observed when co-transplanting selected ESC-derived cardiomyocytes and fibroblasts into the injured heart of syngeneic mice, and no teratoma formation was found. Although transplantation of ESC-derived cardiomyocytes improved heart function, BM cells had no positive effects. Furthermore, no contribution of BM cells to cardiac, endothelial, or smooth muscle neogenesis was detected. These results demonstrate that ESC-based therapy is a promising approach for cellular cardiomyoplasty of impaired myocardial function and provides better results than BM-derived cells.

## *Stem Cell Therapy for Cardiac Regeneration*

Stem cell-based therapies in subacute or chronic phases of myocardial infarction aim at regenerating the myocardium. Several approaches have been used.

### Regeneration of the Chronic Myocardial Infarcts by HSC Therapy

The medications and interventional therapies available so far are intended only to limit further damage to the heart. In contrast, HSC therapy has the potential not only to limit further damage, but to regenerate heart function. In IACT study, 18 consecutive patients with chronic myocardial infarction (up to 8 years after a heart attack) were treated by the intracoronary infusion of autologous bone marrow mononuclear cells and compared with a representative control group without cell therapy (Strauer et al. 2005). After 3 months, infarct size was reduced by 30% in the transplantation group with improvement of the left ventricular function by 15%, whereas no significant changes were observed in the control group. Percutaneous transluminal coronary angioplasty alone had no effect on left ventricular function. After bone marrow cell transplantation, there was an improvement of maximum oxygen uptake and of regional $^{18}$F-fluoro-deoxy-glucose uptake into infarct tissue. These results demonstrate that functional and metabolic regeneration of infarcted and chronically damaged tissue can be achieved in humans by bone marrow stem cell transplantation. This therapy is safe, similar to transfusion of a patient's own blood and has no side effects. It requires only bone marrow puncture and cell aspiration with subsequent stem cell preparation. Larger trials are now underway that could verify the findings.

### Human Mesenchymal Stem Cells for Cardiac Regeneration

Mesenchymal stem cells (MSCs), derived from the bone marrow, are able to differentiate into cardiomyocytes in vitro. When implanted into myocardium, MSCs can undergo milieu-dependent differentiation and express cardiomyogenic phenotypes in vivo. To investigate the potential of human mesenchymal stem cells (hMSCs) to undergo differentiation to a muscle phenotype and to regenerate muscle tissue in the failing heart, Osiris Therapeutics Inc has developed culture conditions to induce myogenic differentiation of hMSCs and has also implanted hMSCs into the hearts of animal models. In an open-heart procedure, the Osiris team has implanted hMSCs by a needle injection directly into the ventricle wall of athymic rats. The hMSCs were labeled with a vital dye and were identified with antibodies that react with human cells (but not the host animal tissue). It has been verified that hMSCs engraft in the heart at various times following introduction and the expression of muscle specific proteins has been demonstrated. Results from an animal study conducted at Johns Hopkins University (Baltimore, MD) showed that adult MSCs (developed according to method of Osiris Therapeutics) can be used effectively to treat myocardial infarction in pigs (Amado et al. 2005). MSCs taken from another pig's bone marrow, when injected into the animal's damaged heart, are able to restore the heart's function to its original condition. The cells were injected

directly into the heart muscle using a specialized catheter (Biocardia Inc) inserted through a tiny puncture in an artery, a procedure similar to other cardiac catheterization techniques. Use of this catheterization technique was shown to be safe and effective. Its use increases the options for delivering stem cell therapy in the future; most existing studies use intravenous injections. If further animal studies and human clinical trials prove equally successful, the investigators believe this could be a new, widely applicable treatment to repair and reverse the damage done to heart muscle that has been infarcted, or destroyed, after being deprived of its blood supply. Using MSCs also avoided potential problems with immunosuppression, in which each animal's immune system might have attacked SCs from sources other than itself. Because they remain in an early stage of development, MSCs do not trigger an immune response, unlike what would happen if more developed SCs were used. Among many benefits of this approach are that adult SCs are readily available, that is, they can be extracted from the patient, no donor is required, and the cells can be simply reproduced if more are needed. This study forms the basis of a clinical trial of autologous MSCs in patients with myocardial infarction.

In 2001, MSCs from a man's bone marrow were microinjected directly into the tissue of his heart during coronary artery bypass grafting at the Clinic of Cardiac Surgery in Rostock, Germany. No complications were detected after the procedure, and the physicians have performed the same operation on other patients. In 2005, ten clinical trials started in the USA to test MSCs (Osiris' Prochymal) for acute myocardial infarction.

In 2006, University of Rochester (Rochester, NY) started a multicenter randomized, double-blind, placebo-controlled, phase I clinical trial with patients randomized to receive either an injection of 0.5 million, 1.6 million, or 5.0 million cultured adult MSCs (Osiris Therapeutics' Prochymal) per kilogram of body weight, or placebo. All patients received standard treatment, including techniques to maximize blood flow to damaged areas, pain relief, oxygen, anticoagulants, β-blockers, nitrates, ACE-inhibitors, and advice on reducing risk factors. Trial entry occurred within 10 days of first heart attack, and patients were followed for 2 years afterward. The results, published in 2009, are described later in this chapter.

A key issue with the use of MSCs is the development of a suitable system for their delivery to the site of infarction. One delivery system uses a fibrin-based patch to entrap cells during polymerization. This delivery vehicle has many advantages; however the mechanical properties and the limited capacity for tailoring cell response may restrict its application. A PEGylated fibrin patch for MSC transplantation was developed by modifying fibrinogen with the benzotriazole carbonate derivative of PEG to create secondary crosslinking (Zhang et al. 2006). This PEGylated fibrin patch increases MSC viability and produces phenotypic changes in MSCs consistent with endothelial cells.

### In Vivo Tracking of MSCs Transplanted in the Heart

In vivo trafficking of allogeneic MSCs colabeled with a radiotracer and MRI contrast agent has been dynamically determined in acute myocardial infarction by use of the high sensitivity of a combined SPECT/CT scanner (Kraitchman et al. 2005).

Redistribution of the labeled MSCs after intravenous injection from initial localization in the lungs to nontarget organs such as the liver, kidney, and spleen was observed within 24–48 h after injection. Focal and diffuse uptake of MSCs in the infarcted myocardium was already visible in SPECT/CT images in the first 24 h after injection and persisted until 7 days after injection and was validated by tissue counts of radioactivity. In contrast, MRI was unable to demonstrate targeted cardiac localization of MSCs in part because of the lower sensitivity of MRI. Noninvasive radionuclide imaging is well suited to dynamically track the biodistribution and trafficking of MSCs in the heart following myocardial infarction. In a further study, the authors showed that a more advanced technique used with MRI, called inversion recovery with on-resonant water suppression (IRON), could be used to monitor iron-labeled stem cells. This technique showed that stem cells were incorporated into the heart tissue itself, mostly in the peri-infarction zone. Measurements of the heart's pumping function also improved in the same region. MRI tracking of MSCs can be used as an alternative to surgical biopsy to verify that cell-based therapies reached damaged areas of the heart and were able to induce repair and improve heart function. With IRON, conventional MRI technology is adapted to reveal images of ever smaller numbers of cells, avoiding image artifacts that mimic the appearance of iron-labeled cells. Scientists were able to visualize metal objects, which previously appeared as dark spots on the MRI screen, by suppressing the vast majority of the conventional image produced from water molecules (or so-called on-resonant signal), the most common substance in the body. The ready availability of MRI machines means that IRON could be widely introduced into clinical practice within a relatively short time.

### MSCs for Hibernating Myocardium

In 2006, scientists at the University at Buffalo (Buffalo, NY) started to investigate the potential of bone marrow-derived adult stem cells to treat the serious heart malfunction known as hibernating myocardium, which is a condition in which heart cells that have experienced reduced blood flow over an extended period of time due to narrowed coronary arteries adapt to this deprivation by downregulating metabolism while remaining functionally viable. Previous work at the university's Center for Cardiovascular Research employing the center's novel swine model of hibernating myocardium has shown that restoring normal blood flow to these "hibernating" regions improves function. However, these results also found that cells in the left ventricle of the heart often do not return to normal, leaving the heart compromised. The swine model was used to investigate whether transplanting the model's own bone marrow MSCs can change the myocardial adaptive responses and improve the function of the hibernating myocardium. In the long term, the translation between the MSC-based therapy in the porcine hibernating myocardium and regenerative medicine for humans with chronic coronary artery disease will lead to optimized MSC therapeutics that can be of clinical value. The research was carried out in two phases. During the first phase, investigators conducted extensive studies of the characteristics and potential of the targeted stem cells, including research on the

influence of aging on the potency of MSCs because hibernating myocardium usually does not occur in young persons. The second phase of the project involved injecting the stem cells into swine with hibernating myocardium. The researchers tracked the cells' progress to evaluate their feasibility, and determine if cells engineering for enhanced survival, blood vessel regeneration, and "homing potential" (the tendency to migrate properly to the heart rather than elsewhere) can better improve blood flow and tissue function in hibernating myocardium.

#### Simultaneous Transplantation of MSCs and Skeletal Myoblasts

Simultaneous transplantation of cocultured autologous MSCs and skeletal myoblasts improves ventricular function in an experimental model of dilated cardiomyopathy caused by Chagas disease, which is characterized by diffuse fibrosis and impairment of microcirculation (Guarita-Souza et al. 2006). There was improvement in ventricular function 4 weeks following this procedure. Cellular transplantation is thus emerging as a promising strategy for the treatment of postinfarction ventricular dysfunction of Chagas disease. Whether its beneficial effects can be extended to other cardiomyopathies remains an unexplored question.

## Transplantation of Genetically Modified Cells

### *Transplantation of Genetically Modified MSCs*

The poor viability of MSCs at the transplanted site often hinders their therapeutic potential. In a trial designed to address this problem, a nonviral vector of cationized polysaccharide was introduced for genetic engineering of MSCs (Jo et al. 2007). Spermine-dextran was internalized into MSCs by way of a sugar-recognizable receptor to enhance the expression level of plasmid DNA. When genetically engineered by the spermine-dextran complex with plasmid DNA of adrenomedullin (AM), MSCs secreted a large amount of AM, an anti-apoptotic and angiogenic peptide. Transplantation of AM gene-engineered MSCs improved cardiac function after myocardial infarction significantly more than MSCs alone. Thus, this genetic engineering technology using the nonviral spermine-dextran is a promising strategy to improve MSC therapy for ischemic heart disease.

### *Transplantation of Cells Secreting Vascular Endothelial Growth Factor*

Vascular endothelial growth factor (VEGF) is considered to stimulate endothelial cell proliferation and induce angiogenesis in vivo. Gene therapy has been performed by direct myocardial gene transfer of naked DNA-encoding VEGF in myocardial ischemia. A research team at Yamagata University School of Medicine

(Yamagata City, Japan) has transplanted H9C2 cells transfected with VEGF factor gene to the infarcted myocardium in rats. After 2 weeks, the rats were sacrificed and an analysis of their heart tissue was conducted. A monoclonal antibody (MAb) cell marker showed profuse neovascularization around the transplanted cells in the infarcted myocardium. The investigators concluded that H9C2 cells genetically modified with the vascular endothelial growth factor gene and subsequently transplanted into the injured hearts of rats survived and created new areas of vascular cell growth. This approach may hold promise for future clinical use.

### *Transplantation of Genetically Modified Bone Marrow Stem Cells*

Researchers at National University Hospital, Singapore have genetically modified human bone marrow stem cells for cardiac transplantation. The modified cells were implanted into live mice last year but the results of the study are not published as yet. It is proposed that when surgeons open the chest cavity in a heart bypass operation, bone marrow from the breastbone can be collected, converted into heart muscle cells in 4–6 h and then injected into the heart at the end of the operation. This approach overcomes the drawback of using myoblasts from skeletal muscle that are difficult to extract and take several days to grow in the laboratory prior to transplantation.

## Cell Transplantation for Congestive Heart Failure

Congestive heart failure (CHF) is a condition in which areas of the heart are failing because of the presence of scarred, inelastic tissue. The human heart possesses a CSC compartment, and CSC activation occurs in response to ischemic injury. The loss of functionally competent CSCs in chronic ischemic cardiomyopathy may underlie the progressive functional deterioration and the onset of terminal failure (Urbanek et al. 2005). CSC pool in the dog is characterized by undifferentiated cells that are self-renewing, clonogenic, and multipotent. In experimental studies, myocardial reconstitution resulted in a marked recovery of contractile performance of the infarcted heart (Linke et al. 2005). Thus, the activation of resident primitive cells in the damaged dog heart can promote a significant restoration of dead tissue, which is paralleled by a progressive improvement in cardiac function. These results suggest that strategies capable of activating CSC pool, the growth reserve of the myocardium, may be important in cardiac repair after ischemic injury.

The management of CHF is mostly medical. Current treatments for cardiovascular disease prevent heart attack from occurring and/or alleviate its after-effects, but they do not repair the damaged muscle that results, leaving sizably dead portions of heart tissue that lead to dangerous scars in the heart resulting in heart failure. Myoblast as well as SC transplants have been used to repair the damage.

Currently several clinical trials are in progress to test the usefulness and safety of various types of cells in CHF. These are listed at the NIH web site (http://www.clinicaltrials.gov) and a selection is shown here:

- ALCADIA: Intramyocardial injection of CSCs
- CADUCEUS: Intracoronary injection of cardiosphere-derived stem cells
- Cardio 133: Intramyocardial injection of CD133+ stem cells
- Combined CABG and stem cell therapy for heart failure using intramyocardial BMMSCs
- FOCUS Study: Intramyocardial injection of MNCs
- IMPACT-CABG Trial: Intramyocardial injection of CD133+ stem cells
- REVITALIZE: Intracoronary injection of MNCs
- SCIPIO: Intracoronary injection of CSCs
- Surgical treatment with intramyocardial injection of CD34+ autologous stem cells
- TAC-HFT: Transendocardial injection of hMSCs and hBMCs

## *Myoblasts for Treatment of Congestive Heart Failure*

Skeletal myoblast transplantation in a postmyocardial infarction scar experimentally improves left ventricular ejection fraction. Short-term follow-up studies have suggested that a similar benefit could clinically occur despite an increased risk of cardiac arrhythmias. The long-term follow-up of the first worldwide cohort of grafted patients operated on with use of autologous skeletal muscle myoblast transplant and bypass surgery in 2000–2001 (Hagege et al. 2006). In this cohort of severe heart failure patients, both clinical status and ejection fraction stably improve over time with a strikingly low incidence of hospitalizations for heart failure and the arrhythmic risk can be controlled by medical therapy and/or on-request automatic cardiac defibrillator implantation.

Combined transplantation of skeletal myoblasts and angiopoietic progenitor cells results in ventricular function improvement, reduction of scar size, and myocardial apoptosis, and increased neoangiogenesis in chronic ischemia induced experimentally in rats (Bonaros et al. 2006). Clinical studies are warranted to prove this new therapeutic concept. Grafting of skeletal myoblast sheets attenuated cardiac remodeling and improved cardiac performance in dogs (Hata et al. 2006). Feasibility and effectiveness of this method in a large animal model suggests that it is an innovative and promising strategy for treating patients with end-stage dilated cardiomyopathy.

## *Injection of Adult Stem Cells for Congestive Heart Failure*

Results of first prospective randomized trial of injecting adult stem cells into the damaged heart tissue of people with severe CHF, presented by the University of Pittsburgh's McGowan Institute for Regenerative Medicine in 2004, showed

significant improvement of the heart function. Ten of the patients in the study had only cardiac bypass surgery and the other ten had bypass surgery and stem cells injected into their damaged heart tissue during the surgery. Before surgery, the patients who received the stem cells in addition to bypass surgery had an average ejection fraction of 29.4%, while those who had bypass surgery alone had an average ejection fraction of 30.7%. In severe CHF ejection fraction (a standard measure of heart function determined by the total amount of blood pumped out by the left ventricle with each heart beat) is less than 35%. A person is considered to have a good heart function when the ejection fraction is at least 55%. Six months after surgery, the stem cell patients had an average ejection fraction of 46.1% compared with 37.2% for those who had surgery alone. These results will encourage an aggressive pursuit of cellular therapies as an option for CHF. It will change the current approach, which is largely palliative, to one that is truly regenerative.

Further results from a prospective randomized trial by the University of Pittsburgh in 2005 showed that patients with severe CHF who had not responded to other treatments showed markedly improved heart function following a minimally invasive procedure in which autologous bone marrow stem cells were injected directly into the heart. This approach to cell therapy is feasible for the estimated 40% of CHF patients whose disease is unrelated to coronary blockages and who therefore cannot benefit from bypass procedures. Further trials have been conducted that involve giving stem cells to patients who are being implanted with heart assist devices. When a donor heart becomes available for transplantation, the native heart would be removed, allowing investigators the rare opportunity to look at the heart in its entirety and to more closely examine the effects of the stem cells. Further clinical trial will determine the safety and feasibility of injecting a patient's own HSCs directly into the heart in patients with ischemic heart disease who are scheduled for off-pump (beating heart) CABG surgery. In addition to assessing the safety and feasibility of using a patient's own stem cells as a potential therapy for heart disease, investigators are also trying to determine just how many stem cells are needed to produce the best results.

## *AngioCell Gene Therapy for Congestive Heart Failure*

AngioCell™ (Angiogene) program is a pioneering recuperative approach that aims to improve the function of damaged tissue, rather than merely treat the symptoms of the disease. AngioCell is intended to restore the contractility of the heart tissue with a cell therapy treatment that transplants muscle precursor cells loaded with a gene to enhance blood vessel growth. The therapy also improves the blood flow and oxygen uptake of the damaged heart by stimulating the growth of new blood vessels in the myocardium, through a process of therapeutic angiogenesis.

Treating CHF with AngioCell begins with a small skeletal muscle biopsy taken from the patient 2 weeks before surgery. Using a specialized technology process,

these cells are multiplied and genetically treated with Angiogene's proprietary gene, to increase their angiogenic potential. These Angiocells are then implanted into the same patient in the infarcted area of the heart – an autologous graft. The presence of these muscle cells greatly increases the heart's cardiac function, while the angiogenic activity allows better oxygen and nutrient supply to the grafted area, thereby increasing the beneficial effects of the implanted cells. This process is called angiomyogenesis. Because the procedure is an autologous cell transplant, there is no rejection, no immunosuppression, no risk of disease transmission, and no ethical controversy. Use of skeletal muscle cells has the following advantages:

- Easy to extract, purify, and expand
- Ability to colonize scar tissue
- Demonstrates great elasticity
- Long-term survival of implanted cells

## *Stem Cell Therapy for Dilated Cardiac Myopathy*

Dilated cardiac myopathy (DCM) is a chronic cardiac disease that leads to enlargement of the heart and reduces pump function to a point that normal blood circulation cannot be maintained. Typically, patients with DCM present with symptoms of CHF, including limitations in their physical activity and shortness of breath. DCM often represents the end stage of chronic ischemic heart disease in patients who have experienced multiple heart attacks. Patient prognosis depends on the stage of the disease but is characterized by a high mortality rate. Other than heart transplant, there are no effective long-term treatment options for end-stage patients with this disease. In the USA alone, 120,000–150,000 persons currently suffer from this disease.

Scientific and early clinical evidence suggest that high doses of stem and progenitor cells may possibly slow down or reverse disease progression in the heart of DCM patients. Aastrom's Tissue Repair Cells (TRCs), a proprietary product containing large numbers of stem and progenitor cells derived from a small sample of the patient's own bone marrow, can be used as a therapeutic to induce heart tissue regeneration in these patients. If successful, TRC treatment may eliminate or delay the need for a heart transplant. TRC received an orphan drug designation from the FDA in 2007 as a potential new treatment option for patients with DCM, and is conducting clinical trials. In July 2010, Aastrom initiated an extension study for control patients from the its ongoing open-label phase II IMPACT-DCM clinical trial in patients with DCM, which is designed to offer control-group patients from the IMPACT-DCM trial the opportunity to receive treatment with an expanded mixture of their own bone marrow-derived stem and progenitor cells after completing at least 6 months of follow-up. The initiation of this extension study follows a positive review of safety and efficacy data by the IMPACT-DCM data safety monitoring board for the first 20 patients who participated in the IMPACT-DCM trial.

## Role of Cell Therapy in Cardiac Arrhythmias

Cardiac arrhythmias are a leading cause of morbidity in the Western hemisphere. The risk of developing malignant ventricular tachyarrhythmias is associated with the extent of myocardial injury and is believed to be the primary cause of approximately 50% of all cardiovascular deaths. Bradycardia and heart block, which can result from the normal aging process, further add to the morbidity associated with cardiac arrhythmias. About 3 million people worldwide carry implanted electromechanical pacemakers, and each year about 600,000 pacemakers are implanted.

Conventional medical therapy is predominantly palliative treatment and commonly fails to impede and prevent the morbidity and mortality associated with cardiac arrhythmias. Radiofrequency catheter ablation of ischemic ventricular tachycardias is considered adjuvant therapy rather than curative. The implantation of defibrillators and pacemakers, while generally effective, do have problems which include: (1) implantation of a electromechanical device and its need for replacement every 4–7 years, (2) surgical and mechanical complications resulting from the implantation of the device, (3) negative physical and psychological effects of an implanted mechanical device, (4) a prevalent need to use concurrent antiarrhythmic therapy and/or radiofrequency modulation/ablation, and (5) a relatively high cost. Therefore, there is a need to develop alternative therapies for treatment of conduction abnormalities that overcomes the negative aspects of current treatment methods. Electrophysiological studies of pluripotent ESCs show that they functionally express several specialized ion channels indicating potential applications in rhythm disturbances of the heart. Genetically engineered cell grafts, transfected to express potassium channels, can couple with host cardiomyocytes and alter the local myocardial electrophysiological properties by reducing cardiac automaticity and prolonging refractoriness (Yankelson et al. 2008).

### *Atrioventricular Conduction Block*

Cardiomyocytes, developed from hESCs in vitro, can act as biological pacemakers and restore myocardial mechanical function when transplanted into the pacemaker region of pig hearts with a slow heart rate due to atrioventricular conduction block (Kehat et al. 2004). Long-term electromechanical integration between host and donor tissues occurred at several levels. This proof-of-concept study suggests the use of excitable cell grafts as a biological alternative to implantable pacemaker devices. The technique could also be used to repair cardiac muscle tissue damaged during myocardial infarction. It remains to be proven that a biological pacemaker will function nonstop for years in a sick heart and would not be rejected by the recipient's immune system.

Electronic pacemakers, currently used for cardiac arrhythmias, cannot react the way the heart's own pacemaker normally does, for example, raising the heart rate in response to physical exertion. Efforts are being made to restore the heart's ability

to pace itself by putting pacemaker genes permanently into cells in parts of the heart that are not beating properly. The aim is to make a biological pacemaker. The technique, using adenoviral vector-mediated gene transfer in dogs with slow hearts, restored normal 60 beats per minute in parts of the dogs' hearts that had been beating 25–40 times per minute. The technique carries risks associated with viral vector-mediated gene therapy and the results are not permanent. The genetic changes can be made permanent by using stem cells that incorporate the genes without use of viral vectors. This technique has been shown to work. The cellular pacemaker can be turned off, if it becomes hyperactive, by using ivabradine – an approved drug in Europe to slow the heart rate. However, in trials on humans, the cellular pacemaker would only be used alongside an electronic pacemaker, as it would be unethical for researchers to withhold a known effective treatment while testing a new unproven technology.

### Genetically Engineered Cells as Biological Pacemakers

Genetically engineered heart cells derived from hESCs that beat on their own accord, indicate the presence of pacemaker cells. When implanted into the hearts of guinea pigs, these cells trigger regular beating of the heart itself (Xue 2005). These might one day be a promising biological alternative to the electronic pacemakers. The implanted cells also respond appropriately to drugs used to slow or speed the heart rate, which electronic pacemakers cannot do. However, many challenges need to be overcome before this technique could be used for patients.

Guidant Corporation is collaborating with Columbia University Medical Center (New York City) and Stony Brook University to study a technique that involves pacing of the heart by implantation of genetically modified adult human MSCs. Preclinical studies showed that the engineered cell stimulate the heart muscle cells by providing an ionic current, generating a heart beat similar to that of the heart's natural pacemaker. Through this collaboration, the researchers hope to explore the development of a cell-based biological pacemaker.

The latest studies from Columbia University show that the pacemaker current, mediated by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, contributes to the initiation and regulation of cardiac rhythm. Through genetic engineering techniques, a small fraction of heart muscle cells are turned into specialized pacing cells that have a faster, regular, beating pattern, creating a biological pacemaker. This gene family contributes to normal pacemaker function in the heart, and biological pacemakers aim to replicate and enhance this functionality when the normal process fails. Previous experiments creating HCN-based biological pacemakers in vivo found that an engineered HCN2/HCN1 chimeric channel (HCN212) resulted in significantly faster rates than HCN2, interrupted by 1–5 s pauses. To elucidate the mechanisms underlying the differences in HCN212 and HCN2 in vivo functionality as biological pacemakers, newborn rat ventricular myocytes overexpressing either HCN2 or HCN212 channels were studied (Zhao et al. 2009). The HCN2- and HCN212-overexpressing myocytes manifest similar voltage dependence, current density, and sensitivity to saturating cAMP concentrations, but

HCN212 has faster activation/deactivation kinetics. Different HCN channels were expressed in heart cells. Channel parameters and the effect on spontaneous rate were measured, and the channel parameters were entered into a computer simulation to explore their contribution to the rate effect. The biological pacemaker appears to respond to the body's signals in the same manner as the heart's native pacemaker, yet it requires no batteries or replacement of electrical stimulator leads, and could last a lifetime. These results illustrate the benefit of screening HCN constructs in spontaneously active myocyte cultures and may provide the basis for future optimization of HCN-based biological pacemakers for cardiac arrhythmias. In addition, the HCN-expressing heart culture system could serve as a screening assay for new pharmacological agents to regulate heart rate.

## *Ventricular Tachycardia*

Ventricular tachycardia (VT) refers to a rapid heartbeat originating in the ventricles, where the rate is anywhere from 100 to 250 beats per minute. VT is usually the result of serious damage to the heart muscle, although it occasionally occurs in healthy individuals. Patients at the highest risk for VT are commonly treated with implantable defibrillators. Such patients also require the use of antiarrhythmia drugs in the chronic management of VT as a result of the patient's discomfort resulting from the shocks associated with the firing of the defibrillators. Bioheart is developing MyoCell VT™, a cell-based product that can provide VT patients with a normalized heart function, to accomplish these objectives.

The proteins responsible for the cardiac cell to cell communication and the formation of intercalated disks and gap junctions are Cx 43 and N-Cadherin. Dr. Charles Murry, University of Washington, a Bioheart collaborator, has published on the ability of myoblasts to express Cx 43 and N-Cadherin in vitro. By injecting genetically modified myoblasts that have the ability to electromechanically couple in the area of VT block, an "electrical bridge" across the VT block can be established and therefore restore normal electrical conduction pathways within the heart. Current research is focused on in vivo models for simulating the overexpression of Cx 43 and N-Cadherin in preclinical settings and alleviating the conduction blockages that form VT.

## *Prevention of Myoblast-Induced Arrhythmias by Genetic Engineering*

Skeletal myoblasts are an attractive cell type for transplantation because they are autologous and resistant to ischemia. However, clinical trials of myoblast transplantation in heart failure have been plagued by ventricular tachyarrhythmias and sudden cardiac death. Previous research from animal transplants showed that heart tissue regrowth produced a mix of skeletal and heart muscle. The pathogenesis of arrhythmias following myoblast transplant is poorly understood, but may be related to the

fact that skeletal muscle cells, unlike heart cells, are electrically isolated by the absence of gap junctions, or protein connections between cell membranes that allow neighboring cells to communicate with each other through the exchange of ions and other electrical signals. These arrhythmias could be terminated by nitrendipine, an L-type calcium channel blocker, but not by the Na channel blocker lidocaine (Abraham et al. 2005). In gene therapy experiments, production of connexin 43 was increased by injecting a viral vector carrying the gene that codes for the gap-junction protein into the cultured cells. Genetic modification of myoblasts to express the gap junction protein connexin 43 decreases arrhythmogenicity in hearts transplanted with myoblasts. Combining gene therapy to replace connexin 43 along with myoblast transplants may prevent the development of potentially fatal arrhythmias in patients.

## ESCs for Correction of Congenital Heart Defects

Researchers at Memorial Sloan-Kettering Cancer Center in New York have shown that ESCs, injected into embryos of mice genetically predisposed to develop a lethal cardiac defect, prevented the development of this disorder (Fraidenraich et al. 2004). Previous studies had demonstrated a relationship between the presence of a specific protein called Id during embryonic growth and the normal development of capillaries and blood vessels. Two important molecules are implicated in signaling from the ESCs to the Id knock-out cells: insulin-like growth factor 1 (IGF-I) and WNT5a. The former molecule is a long-range acting factor, and the latter is a short-range factor and a member of the family of WNT proteins. Both molecules are implicated in heart development and cancer. Mice engineered without Id, "Id knock-out" mice, display severe cardiac defects and die at mid-gestation. Following injection of ESCs into these mice, not only did the daughter ESCs incorporate into the defective embryonic heart but they also released biological factors preventing neighboring heart cells from developing into defective tissue. The result was that 50% of the mice fated to die in utero were born with healthy hearts. The authors demonstrated that IGF-I injected into the mother can cross the placenta and influence fetal cardiac development in the Id knock-out embryo. The Id knock-out embryos were born, but with partial rescue of cardiac defects and abnormal gene expression profiles. As a result, Id knock-out pups whose mothers were manipulated bypassed mid-gestation lethality, although they died during the first 2 days of life. On the other hand, WNT5a had the ability to correct the abnormal gene expression profiles of the Id knock-out hearts to normal levels. These two mechanisms (long- and short-range action) in conjunction may account for the full correction of the cardiac defects.

## Cardiac Progenitors Cells for Treatment of Heart Disease

Because fully developed heart cells do not divide, the organ is viewed as incapable of regeneration after injury but this belief is now challenged. Until now there has been little evidence for native cardiac precursor cells in the postnatal heart but a

recent study shows that heart contains cells that can divide and mature after birth, which might allow the organ to regenerate itself. Cardiac progenitor cells (islet-1) have been identified in postnatal rat, mouse, and human myocardium (Laugwitz et al. 2005). These are very rare cells, which accounts for why they have not yet been reported. Only a few hundred progenitor cells remain in the heart after birth, and this number decreases with age. Unlike stem cells, which have a seemingly unlimited capacity for self-renewal, progenitor cells undergo a finite number of divisions. But both types of cells have potential for use in repairing heart damage. Cardiac progenitor cells, however, have one considerable advantage over stem cells: scientists can easily coax them into becoming fully specialized heart-muscle cells, without chemical or hormonal stimuli. The next research steps with the islet-1 cells will be cellular transplantation in living animals to study their role in endogenous repair after cardiac injury. Since these rare cardiac progenitor cells are found in regions of the atrium that are normally discarded during routine cardiac surgery, the discovery raises the possibility that an individual could receive their own cardiac stem cells to correct a wide spectrum of pediatric cardiac diseases. If the cells maintain pacemaker function when placed in the intact heart, they might serve as biological pacemakers for infants born with heart block, which could also be valuable.

Progenitor cells in culture have been shown to progress to organized contracting myocytes after chemical and electrical stimulation (Abilez et al. 2006). Incorporation of such cells into existing methods of producing endothelial cells, fibroblasts, and scaffolds may enable production of improved tissue-engineered vascular grafts. By creating a tissue-engineered blood vessel grown from a patient's own stem cells, the problem of graft rejection could potentially be eliminated.

## Autologous Stem Cells for Chronic Myocardial Ischemia

In 2006, the University of Pittsburgh School of Medicine started a novel phase II clinical trial to study if a patient's own stem cells can treat chronic myocardial ischemia (CMI) due to severe coronary artery disease, who are currently on maximal medical therapy and are not suitable candidates for conventional procedures to improve blood flow to the heart, such as angioplasty, stents or coronary artery bypass surgery. Baxter Healthcare Corporation's ISOLEX 300i Magnetic Cell Selection System is used to select the patient's own CD34+ stem cells that are under investigation of this trial. The stem cells are injected directly into the heart muscle by NOGA® XP cardiac navigation system, which enables injection of cells into a specific area of the heart muscle by a procedure not much more invasive than a cardiac catheterization. All patients receive granulocyte colony stimulating factor by subcutaneous injections, which helps to release blood-forming CD34+ cells from the subject's bone marrow into the bloodstream, investigating the injection of autologous CD34+ cells into the hearts of CMI patients. Although data from the preceding phase I trial have not been fully analyzed, the therapy appeared to be well-tolerated and no serious adverse events directly related to the stem cell therapy were

reported. Two thirds of phase I study subjects reported feeling better with reductions in chest pain and improved exercise capacity during the early stage of the trial.

NX-CP105 (Neuronyx), an investigational biologic of human adult bone marrow-derived somatic cells (hABM-SC), is being developed for the repair of cardiac tissue following acute myocardial infarction. A phase I trial is assessing the safety following NX-CP105 injections directly into the damaged heart tissue using a minimally invasive catheter. An electromechanical mapping system is integrated into the catheter to locate the area of damage and allow for precise delivery of cells to the damaged area of the heart. Patients will be monitored for heart function and changes in the size of the damaged heart area.

## Role of Cells in Cardiovascular Tissue Engineering

### *Construction of Blood Vessels with Cells*

In blood vessel tissue engineering, the focus is on small diameter vascular grafts for coronary bypass surgery. A key issue for a blood vessel substitute is that of cell source. It is important to provide an "endothelial-like" inner lining to interface with the flowing blood. Mature endothelial and smooth muscle cells have been isolated and cultivated for the ex vivo assembly of functional autologous vessel grafts in animal models. Translation of this technology to the creation of autologous human grafts is limited by the expandability of mature vascular cells from patients with diseased blood vessels.

Scientists at the University of Queensland (Brisbane, Australia) have produced a "designer" artery in the peritoneal cavity from cells of the same animal into which it is grafted subsequently. These arterial substitutes, grown around removable tubes, are composed of functional living cells that are antigenically acceptable and are lined by a layer non-thrombogenic cells. Studies in which irradiated female mice were transfused with bone marrow from a congenic strain of male mice showed that the myofibroblasts were originally derived from hemopoietic cells of the donor male and thus most likely from the peritoneal macrophages, were visible on the surface of the tubing during the first few days after implantation. These studies raise the possibility that arteries of predetermined length and diameter can be grown in the peritoneal cavity.

Multipotent SCs, easily obtained from adult tissues, can also be useful for vascular reconstruction. Distinct populations of adult SCs isolated from bone marrow as well as skeletal muscle, have the potential to give rise to vascular endothelial in vivo during injury-induced neovascularization. Selected adult SCs can serve as hematopoietic progenitors that can contribute to vascular regeneration after injury. SCs that preferentially engraft into vascular endothelium and smooth muscle will be uniquely useful for building blood vessel layers for vascular tissue engineering applications. Researchers are trying to grow test tube arteries from skin stem cells for use in heart bypass operations.

### *Targeted Delivery of Endothelial Progenitor Cells Labeled with Nanoparticles*

Circulating endothelial progenitor cells (EPCs) are involved in physiological processes such as vascular re-endothelialization and postischemic neovascularization. However, the success of cell therapies depends on the ability to deliver the cells to the site of injury. Human EPCs labeled with iron oxide superparamagnetic nanoparticles, could be targeted to site of common carotid artery injury in rats using an externally applied magnetic device (Kyrtatos et al. 2009). There was a fivefold increase in EPC localization at the site of vascular injury at 24 h after implantation in vivo.

### *Fetal Cardiomyocytes Seeding in Tissue-Engineered Cardiac Grafts*

The synthetic materials currently available for the repair of cardiac defects are nonviable, do not grow as the child develops, and do not contract synchronously with the heart. To overcome this problem Genzyme and surgeons at the Toronto General Hospital (Toronto, Canada) developed a beating patch by seeding fetal cardiomyocytes in a biodegradable scaffold in vitro. A gelatin patch was used to replace the right ventricular outflow tract in syngeneic rats. The seeded cells survived in the right ventricular outflow tract after the scaffold dissolved 12 weeks after implantation. In addition, the unseeded patches encouraged the ingrowth of fibrous tissue as the scaffold dissolved and the patches remained completely endothelialized. Clinical trials are planned with the autologous bioengineered cardiac graft.

### *UCB Progenitor Cells for Engineering Heart Valves*

Engineering of biologically active heart valve leaflets using prenatally available human UCB-derived progenitor cells as the only cell source has been demonstrated (Schmidt et al. 2006). Wharton's Jelly derived cells and UCB-derived endothelial progenitor cells were subsequently seeded on biodegradable scaffolds and cultured in a biomimetic system under biochemical or mechanical stimulation or both. Depending on the stimulation, leaflets showed mature layered tissue formation with functional endothelia and extracellular matrix production comparable with that of native tissues. This demonstrates the feasibility of heart valve leaflet fabrication from prenatal UCB-derived progenitor cells as a further step in overcoming the lack of living autologous replacements with growth and regeneration potential for the repair of congenital malformations.

## Cell Therapy for Peripheral Vascular Disease

Peripheral vascular disease (PVD), usually resulting in lower-limb ischemia, is a major health problem. Individuals with PVD have decreased blood flow to the muscles of the legs, especially during exercise, which causes pain, aching, cramping, or fatigue in the muscles of their legs when they walk. The condition is called intermittent claudication. Various medical and surgical treatments have been tried. For advanced critical limb ischemia (CLI) patients with no other therapeutic options for improving blood flow, amputation of the affected limb is often the only available clinical option. Within 6 months of diagnosis up to 35% of CLI patients require limb amputation and 20% die. Cell therapy is one of the innovative approaches for treatment of PVD and CLI.

### *ALD-301*

ALD-301 (Aldagen Inc) is a potent population of stem and progenitor cells, isolated from patient's bone marrow using Aldagen's technology and is administered via intramuscular injections into the leg of patients with CLI. ALD-301 contains a mixture of all of the stem and progenitor cell types needed for therapy including endothelial, mesenchymal, and neural progenitors. In preclinical testing, ALD-301 has been shown to repair ischemic damage and to significantly out-perform the unfractionated adult bone marrow preparation. Results of phase I/II clinical trials have shown that ALD-301 was well tolerated, and patients in the treatment group were shown to have increased blood flow and improved clinical status. In 2009, Aldagen received an SPA concurrence letter from the FDA for the design of a pivotal phase III clinical trial of ALD-301 for the treatment of CLI, which is expected to commence.

### *Cell/Gene Therapy for PVD*

Ischemia induces the production of angiogenic cytokines and the homing of bone marrow-derived angiogenic cells (BMDACs), but these adaptive responses become impaired with aging because of reduced expression of hypoxia-inducible factor (HIF)-1α. Effect of augmenting HIF-1α levels in ischemic limb has been tested by intramuscular injection of AdCA5, an adenovirus encoding a constitutively active form of HIF-1α, and intravenous administration of BMDACs that were cultured in the presence of the prolyl-4-hydroxylase inhibitor dimethyloxalylglycine to induce HIF-1 expression (Rey et al. 2009). The combined therapy increased perfusion, motor function, and limb salvage in old mice subjected to femoral artery ligation. Homing of BMDACs to the ischemic limb was dramatically enhanced by intramuscular AdCA5 administration. Thus, HIF-1α gene therapy increases homing of

BMDACs to ischemic muscle, whereas HIF-1 induction in BMDACs enhances their adhesion to vascular endothelium, leading to synergistic effects of combined therapy on tissue perfusion.

## *Colony Stimulating Factors for Enhancing Peripheral Blood Stem Cells*

Treatment of PVD involves use of colony stimulating factors to stimulate the bone marrow to release EPCs, which can form new blood vessels or repair damaged ones. In a randomized and blinded clinical trial, patients with PVD were given an injection of either GM-CSF (granulocyte-macrophage colony stimulating factor) or a placebo. The level of EPCs in the blood was measured before, during and after administration of the drug or placebo. The results showed that the GM-CSF treatment increased total WBC count and the total number of circulating EPCs. Patients who received GM-CSF therapy also experienced an improvement in the ability to walk without pain. The placebo group experienced no improvement after therapy. Currently, GM-CSF is approved by the FDA for several uses, including in cancer patients to increase the number of WBC to fight infection after chemotherapy; in healthy individuals serving as bone marrow donors to stimulate the bone marrow to release stem cells; and in patients who have had a bone marrow transplant to increase the number of WBCs. It is still considered experimental, however, for use to increase the level of EPCs in patients with PVD.

In 2007, Beike Biotechnology Co announced that a team at its collaborating hospital in Shenyang, China, completed an open study to assess clinical efficacy, safety, and feasibility of transplantation of autologous peripheral blood stem cells mobilized with G-CSF for patients with peripheral vascular disease of the lower extremities (Xiaofeng et al. 2006). At 12 weeks, primary manifestations, including lower limb pain and coldness, were significantly improved in 137 (90.1%) of the patients; limb ulcers improved or healed in 46 (86.8%) of the 53 patients, while 25 of the 48 (47.9%) patients with limb gangrene remained steady or improved. Angiography before treatment, and at 12 weeks after treatment, was performed in ten of the patients and showed formation of new collateral vessels. No severe adverse effects or complications specifically related to cell transplantation were observed.

## *Intramuscular Autologous Bone Marrow Cells*

Intramuscular autologous bone marrow cells (BMCs) have also been used for the treatment of PVD. Because tissue ischemia is associated with an overwhelming generation of oxygen radicals and negative effects due to perturbed shear-stress,

metabolic intervention with antioxidants and L-arginine could potentially induce beneficial effects beyond those achieved by BMCs. The protective effect of autologous BMCs and vascular protection by metabolic co-treatment (vitamin E added to the chow and vitamin C and L-arginine added to the drinking water) were examined in ischemia-induced angiogenesis in the mouse hind limb, a model of extensive acute peripheral arterial occlusion (Napoli et al. 2005). Intravenous BMC therapy improved blood flow and increased capillary densities and expression of Ki-67, a proliferation-associated protein. This beneficial effect was amplified by metabolic co-treatment, an intervention inducing vascular protection, at least in part, through the nitric oxide pathway, reduction of systemic oxidative stress, and macrophage activation. Therefore, although a cautious approach is mandatory when experimental findings are extended to human diseases, autologous BMCs together with metabolic intervention could be an effective clinical treatment for PVD.

### *Vascular Repair Cell*

Aastrom Biosciences Inc has conducted a prospective, double-blind, randomized phase IIb clinical trial on patients suffering from CLI, the most severe form of peripheral arterial disease. The company is using its Vascular Repair Cell (VRC), which enables patient-specific stem cell product composed of stem and progenitor cells required for tissue regeneration in the human body. A normal dose of VRCs contains significantly more of these key cells than can normally be harvested from a patient and this may be extremely important when treating critical limb ischemia in diabetic patients with severely impaired blood flow that can affect the majority of their lower limb. The primary objective of the clinical trial is to assess the safety of the cell therapy product. Secondary objectives include assessing amputation rates, wound closure and blood flow in the affected limbs, patient quality of life, and the reduction of pain and analgesic use. In November 2010, the interim analysis of all 86 patients enrolled in the RESTORE-CLI clinical trial, 72 of whom were eligible for treatment and completed 12 months of follow-up, shows that the study achieved both its primary safety endpoint as well as primary efficacy endpoint of time to first occurrence of treatment failure. There was a clinically meaningful reduction of 56% in treatment failure events. These results will be used to plan the phase III trials anticipated for 2011.

## Clinical Trials of Cell Therapy in Cardiovascular Disease

Clinical trials of cell therapy, cited in the preceding sections, are shown in Table 7.2.

**Table 7.2** Clinical trials of cell therapy in cardiovascular disease

| Indications | Type of cell and procedure | Trial | Sponsor |
|---|---|---|---|
| Angina pectoris refractory to treatment | Vescell™: autologous angiogenic cell precursors isolated from blood, expanded in the laboratory and injected into the coronary arteries via catheter | Open trial | Theravitae Ltd |
| Angina pectoris (severe and chronic) | Injection into the heart of autologous purified CD-34 cells to spur regrowth of small blood vessels that constitute the microcirculation of the heart | Open trial | Northwestern University (Chicago, IL) |
| Angina pectoris | NOGA XP cardiac navigation system used to inject a patient's own stem cells into his/her heart | Open trial | Cleveland Clinic, Ohio |
| Chronic myocardial ischemia | Autologous cellular therapy (ACT): blood-derived CD34+ stem cells injected directly into specific area of the heart muscle by NOGA® XP catheter | Phase II | University of Pittsburgh Medical Center (Pittsburgh, PA) |
| Chronic myocardial ischemia | t2c001-AMI: intracoronary infusion of autologous bone marrow-derived progenitor cells | Phase I/II | t2cure/Goethe-University (Frankfurt) |
| Chronic myocardial ischemia | Autologous cellular therapy (ACT): blood-derived CD34+ stem cells, mobilized by G-CSF and sorted by Isolex (Baxter), injected directly into specific area of the heart muscle by NOGA® XP catheter. Results: relief of angina and reduced mortality | Phase II, double-blind, placebo controlled | Baxter/Minneapolis Heart Institute, (Minneapolis, MN) Northwestern Mem Hospital (Chicago, IL) |
| Chronic myocardial ischemia | ACT34-CMI in patients who are currently on maximal medical therapy and are not suitable candidates for conventional procedures. CD34+ stem cells isolated using Baxter's ISOLEX 300i magnetic cell selection system | Phase II, double-blind, placebo-controlled | Columbia University Medical Center, New York |
| Chronic myocardial ischemia | PRECISE study evaluates safety and feasibility of Celution stem and regenerative cell processing system for autologous adipose-derived stem cells to be reinjected into damaged heart | Phase II, randomized | Cytori Therapeutics/Hospital Gregorio Maranon (Madrid, Spain) |
| Chronic myocardial ischemia/coronary artery disease | Autologous cellular therapy CD34-chronic myocardial ischemia (ACT34-CMI) to test efficacy, tolerability, and safety of CD34+HSCs | Phase II | Rush University Medical Center (Chicago, IL) |

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| Chronic myocardial ischemia/reduced pumping function | Direct intramyocardial injection of autologous CD133+ bone marrow stem cells in addition to in patients who also had CABG | Phase I/II randomized | Miltenyi Biotec/Heart Centers in Rostock, Berlin, and Hannover |
| Congestive heart failure needing ventricular assist device | Injection of 25–45 million autologous bone marrow-derived stem cells into the diseased myocardium during VAD implant procedure | Open trial on 5–10 patients | University of Pittsburgh Medical Center (Pittsburgh, PA) |
| Coronary artery disease, myocardial infarction, congestive heart failure | MYOHEART (Myogenesis heart efficiency and regeneration trial)<br>Drug: MyoCell™<br>Device: MyoCath™ | Phase I | Bioheart Inc |
| Coronary artery disease, myocardial infarction, congestive heart failure | Intracoronary infusion of stem cells for safety and efficacy studies | Phase I | Amorcyte Inc/Emory University School of Medicine (Atlanta, GA) |
| Coronary artery disease, candidates for bypass surgery | Autologous bone marrow stem cells to be injected directly into open hearts during surgery | Phase I | University of Utah, Salt Lake City |
| Coronary artery disease, unsuitable for treatments such as coronary stenting or bypass surgery | Targeted intracoronary and intramyocardial injection of bone marrow-derived adult HSCs using MYOSTAR™ injection catheter and NOGA® cardiac navigation system (cordis corporation). The results were satisfactory | Pilot trial | University of Vienna Medical Center (Vienna, Austria) |
| Coronary artery disease, severe with myocardial damage | Autologous cultured stem cells delivered by cardiac catheter improved heart failure/cardiac function within 3 months and met safety endpoint of 6 months | Pilot trial on six patients | Mesoblast Ltd/angioblast systems |
| Dilated cardiomyopathy, with heart failure | Autologous stem cell therapy: Cardiac Repair Cells (CRCs), generated from the patient's bone marrow, injected directly into the heart muscle | Open study on patients completed | Aastrom Biosciences/University Hospital (Dusseldorf, Germany) |

(continued)

**Table 7.2** (continued)

| Indications | Type of cell and procedure | Trial | Sponsor |
|---|---|---|---|
| Dilated cardiomyopathy, with heart failure | IMPACT-DCM: autologous CRCs (a mixture of stem and progenitor cells derived from the patient's bone marrow), injected directly into the heart muscle. Positive results in first 20 patients, extension to treat control subjects in July 2010 | Phase II randomized controlled prospective open-label | Aastrom Biosciences & Baylor University Medical Center (Dallas, TX ) /University of Utah (Salt Lake City, UT) |
| Dilated, nonischemic cardiomyopathy | t2c001-AMI: intracoronary infusion of autologous bone marrow-derived progenitor cells | Phase I/II | t2cure/ Goethe-University (Frankfurt) |
| Heart failure | Autologous adult bone marrow-derived mononuclear cells and adult autologous MSCs delivered by Morph®guide catheter | Phase I/II | BioCardia/University of Miami (Miami, FL) |
| Heart failure | C-Cure™: autologous MSCs differentiated into cardiac precursors called cardiopoietic cells | Phase I/II | Cardio3Biosciences Clinical Center, Serbia |
| Ischemic cardiomyopathy | Intramyocardial injection of ALD-201 stem cells for therapeutic angiogenesis | Phase Ib randomized | Aldagen/Texas Heart Institute |
| Myocardial infarction/ acute phase | Intravenous injection of adult MSCs (prochymal) within 10 days of first heart attack, and patients will be followed for 2 years afterward | Phase I, randomized double-blind | University of Rochester Medical Center (Rochester, NY)/Osiris |
| Myocardial infarction/ acute phase | Intravenous injection of adult MSCs (prochymal) within 10 days of first heart attack, and patients will be followed for 2 years afterward | Phase I randomized double-blind | Rush University Medical Center (Chicago, IL)/Osiris |
| Myocardial infarction/ acute phase | Intravenous injection of allogeneic adult MSCs (Prochymal) within 10 days of first heart attack, and patients were followed for 6 month afterward. Excellent results (see text) | Phase I, randomized double-blind | Stem Cell Institute, University of Miami (Miami, FL)/Osiris |
| Myocardial infarction/ acute phase | Injection of HSCs from bone marrow through angioplasty catheter within hours of the heart attack to prevent or delay the onset of heart failure | Phase II double-blind | University College Hospital (London, UK) |
| Myocardial infarction/ acute phase | NX-CP105, a biologic of human adult bone marrow-derived somatic cells, injected directly into the damaged heart tissue using a minimally invasive catheter | Phase I | Neuronyx |

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|---|---|---|---|
| Myocardial infarction/ acute phase | APOLLO study evaluates safety and feasibility of Celution stem and regenerative cell processing system for autologous adipose-derived stem cells delivered through an intracoronary catheter within 36 h following the onset of a heart attack | Phase II, randomized | Cytori Therapeutics/Hospital Gregorio Maranon (Madrid, Spain) and Erasmus Medical Center (Rotterdam, NL) |
| Myocardial infarction/ acute phase | t2c001-AMI: intracoronary infusion of autologous bone marrow-derived progenitor cells | Phase III | t2cure/ Goethe-University (Frankfurt, Germany) |
| Myocardial infarction/ acute phase | Allogenic mesenchymal precursor stem cells injected directly into the heart with a special catheter to prevent congestive heart failure | Phase I | Angioblast systems/Texas Heart Institute |
| Myocardial infarction/ acute phase | Cardiac stem cells, removed by biopsy of patient's heart are multiplied in lab and injected back a month later into the heart via a coronary artery | Phase I | Cedars-Sinai Heart Institute, Los Angeles |
| Myocardial infarction | Ex vivo expansion of autologous skeletal muscle cells and implantation into scarred region of the heart during a coronary artery bypass operation | Phase II enrollment completed | Genzyme/Myosix SA (Paris) |
| Myocardial infarction/ chronic phase | Intracoronary infusion of autologous HSCs from bone marrow. The results showed functional and metabolic regeneration of infarcted and chronically damaged cardiac tissue | Open trial on 18 patients | Heinrich-Heine-University Hospital (Dusseldorf, Germany) |
| Myocardial infarction | Injection of autologous bone marrow stem cells into the infarct border zone in patients who had undergone coronary artery bypass grafting | Open study | University of Rostock (Rostock, Germany) |
| Myocardial infarction | Intravenous injection of adult MSCs from the patient's bone marrow | Phase I | Johns Hopkins/Osiris Therapeutics |
| Myocardial infarction | AMR001: bone marrow derived autologous CD34+ cells delivered by a balloon catheter | Phase I | Amorcyte Inc |
| Myocardial infarction and congestive heart failure | MyoCell™: proprietary transport and injection media, biopsy enzyme disassociation process, myoblast selection process, culturing media, and transplantation into the damaged heart muscle | Phase III | Bioheart Inc/University of Rotterdam, The Netherlands |
| Myocardial ischemia | Injection of autologous HSCs directly into the heart during conventional heart bypass surgery | Open study | University of Pittsburgh Medical Center (Pittsburgh, PA) |

(continued)

**Table 7.2** (continued)

| Indications | Type of cell and procedure | Trial | Sponsor |
|---|---|---|---|
| Myocardial ischemia with heart failure | Skeletal autologous myoblasts | Open study | BichatClaude Bernard Hospital (Paris, France) |
| Peripheral vascular disease/critical limb ischemia (CLI) | Vascular repair cell, a patient-specific stem cell product, containing large numbers of early stage stem and progenitor cells for tissue regeneration | Phase IIa | Aastrom Biosciences Inc |
| Peripheral vascular disease/CLI | ALD-301, a mixture of stem and progenitor cells, isolated from bone marrow using ALDESORT® | Phase I/II completed | Aldagen Inc/Texas Heart Institute |
| Peripheral vascular disease/CLI | Injection of autologous $CD34^+$ cells for symptomatic relief and ischemic wound healing | Phase I/II | Baxter Healthcare/US University Centers |
| Peripheral vascular disease | To assess clinical efficacy, safety, and feasibility of transplantation of peripheral blood stem cells | Open study | Beike Biotech (Shenyang, China) |
| Peripheral arterial disease (PAD) | Allogenic PLacenta eXpanded Mesenchymal cells (PLX)-PAD injection along with UCB | Phase I/II in US, Germany | Pluristem Therapeutics (Israel) |
| Peripheral artery occlusive disease | t2c002-PAOD: infusion of autologous bone marrow-derived progenitor cells | Phase II | t2cure/Goethe-University (Frankfurt) |

## Mechanism of the Benefit of Cell Therapy for Heart Disease

In spite of tremendous activity in cell therapy of cardiac disease, the mechanism for potential therapeutic effect is unclear. A growing body of evidence suggests that the improvement in cardiac function is largely independent of cardiac muscle regeneration. A study provides evidence that bone marrow-derived c-kit+ cells can lead to an improvement in cardiac function in mutant hypomorphic c-kit mice that is independent of transdifferentiation into either cardiac muscle or endothelial cells, but rather is associated with the release of angiogenic cytokines and associated neovascularization in the infarct border zone (Fazel et al. 2006). These findings suggest the potential therapeutic effect of specific paracrine pathways for angiogenesis in improving cardiac function in the injured heart.

## A Critical Evaluation of Cell Therapy for Heart Disease

Cell therapy of heart diseases is promising but has not yet proven itself. Adequate cell-based repair of adult myocardium remains a desirable goal but most cells that are used cannot generate mature myocardium sufficient to promote large functional improvements. ESCs can generate both mature cardiocytes and vasculature, but their use is hampered by associated teratoma formation and the need for an allogeneic source. There is still difference of opinion among the cardiologists if the therapy is ready to go into clinical practice. Several clinical trials of the bone marrow-to-heart approach have been completed and some have shown positive results. The overall degree of improvement in the patients' heart function has been modest. The difficulties of the marrow-to-heart approach indicate that more research is required.

The bone marrow stem cell technique appears to be reasonably safe because the patients are injected with their own cells. Clinical trials are still in progress, for example, at the Texas Heart Institute (Houston, TX). Earlier results were promising. In 2004, two laboratories, one at Stanford University (Palo Alto, CA) and the other at University of Washington (Seattle, WA) were unable to replicate the results claiming transformation of bone marrow stem cells turn into heart tissue. The few that lodged in the heart turned into blood cells in the usual way. According to the clinicians, the technique is safe as several hundred patients have now been treated worldwide without adverse effect and the results so far are encouraging. Most studies, however, show 5–10% improvement in heart function. This may be small but is significant in view of the fact that most of the patients were very sick to start with.

The adult human heart as a source for cardiac stem cells is being further investigated. There is potential for autologous repair or even cardiac regeneration with cells that follow a developmental pathway similar to embryonic cardiac precursors but without the inherent limitations associated with undifferentiated ESCs.

### *Current Status of Cell Therapy for Cardiovascular Disease*

Various types of cells are being used on biodegradables matrices for bioengineering of the heart. This may provide new treatments for cardiac disorders such as heart failure and even for replacement of parts of the heart. Bulk of current research is exploring the role of stem cells in repair and regeneration of the heart. Identification of multipotent progenitor cells in the heart and better understanding of developmental processes relevant to ESCs may facilitate the generation of specific types of cells that can be used to treat human heart disease. Secreted factors from circulating progenitor cells that localize to sites of damage may also be useful for tissue protection or neovascularization. The discoveries in basic science require rigorous testing in animal models to determine those most worthy of clinical trials.

Cardiac cell replacement therapy by using hESC-derived cardiomyocytes has emerged as a promising future approach to regenerate functional myocardium. However, there are still many hurdles to be overcome for the clinical application of these cells. A better understanding of the characteristics of the cardiomyocytes from hESCs not only predicts their behavior after implantation but will also help in the design of future strategies for cardiac regeneration in vivo.

Allogeneic adult bone marrow-derived hMSCs are emerging as preferred cells for ameliorating consequences of MI, and have the advantages of ease of preparation, immunoprivilege, capacity to home to injured tissue, and extensive preclinical support. A double-blind, placebo-controlled, dose-ranging safety trial has shown that intravenous allogeneic hMSCs are safe in patients after acute MI (Hare et al. 2009). Stem cell-treated patients had lower rates of side effects, such as cardiac arrhythmias. Echocardiography showed improved heart function, particularly in those patients with large amounts of cardiac damage.

## Future Directions for Cell Therapy of CVD

As cell therapy for CVD evolves, some issues need to be resolved (Flynn and O'Brien 2011):

- Although there are no serious adverse effects in clinical trials, this has been in the setting of the transplanted cells, many of which do not survive beyond a few hours in the recipient heart. Techniques aimed at prolonging cell survival in the future may increase risks of arrhythmias or and tumorigenesis. Close monitoring for adverse events will be essential.
- The optimal cell dose, cell type, and timing of administration are uncertain, and determination of these variables is important for realizing the beneficial effect of cell therapy.
- Barriers within damaged myocardium, such as inflammation, fibrosis, and insufficient angiogenesis need to be addressed as this milieu is likely to hinder optimal functional integration of transplanted cells.

- Transplanted cells probably undergo changes in endogenous gene expression, which may alter the protein expression or metabolic effects of these cells. Novel methods such as cell imaging and directly labeling cells with tracers may enable direct cell tracking and fate of these cells.

## *Prospects of Adult Stem Cell Therapy for Repair of Heart*

The major focus of stem cell research in cardiology is promoting regeneration of the heart or preventing scar formation. More than 90% of research on using stem cells to repair the human heart involves adult stem cells. Therefore this area of application is not affected by controversy about using hESCs. However, FDA still decides as to which adult stem cell techniques are allowed to go into clinical trials and sets the requirements for more routine use.

One study, sponsored by Baxter Inc, which is reporting successful results in humans, involves harvesting autologous stem cells, purifying them, and injecting them directly into the heart muscle. The stem cells have the surface marker CD34, which means they are capable of growing new blood vessels. This is using repair capabilities of a patient's own body. If everything goes well, this treatment could be widely available in by 2013. The target patient population, consisting of end-stage cardiac patients who have tried all other available therapies, can be as high as one million. So far, there are no significant side effects from this method. However, because it is an invasive surgical procedure in which stem cells are delivered through a catheter, there is a risk of perforation of about 1%. There is also a small risk of blood clotting from the drug, G-CSF, which mobilizes stem cells. Injecting stem cells into the heart muscle carries the theoretical risk of arrhythmia but is very low with CD34 cells in general. In a less invasive technique, under investigation in mice, bone marrow-derived stem cells are injected into skeletal muscles of limbs. The stem cells produce growth factors that travel to the heart, in addition to stimulating the muscle itself to make growth factors that also improved cardiac function. The challenge for translating this method to humans application is that, while each mouse needed only a few million stem cells, each human patient would need close to a billion stem cells for the therapy, which would be far too expensive and logistically difficult. This method could be available clinically in 5 years, after researchers find ways to reduce the required number of cells by a factor of 10 or even 100.

Another therapeutic approach is intravenous administration of adult MSCs obtained from a stem cell bank or a company. Such stem cells may not have the right homing receptors to target to receptors in the cardiac muscle. A method is being developed to chemically modify the surface of MSCs to enhance their targeting to specific sites (Karp and Leng Teo 2009). Results from animal models have shown promising results for targeting sites of inflammation. Once the ZIP code of vessels within a certain tissue is known, the address can be programmed on the surface of the cell. These studies use adult MSCs, which may develop into connective tissue,

lymphatic tissue, and blood vessels. These stem cells are largely interchangeable between patients and may not present rejection problems as organ transplants do. However, as more becomes known about the relatively new field of stem cell therapy, a more specific matching system may be required.

### *Regeneration of Cardiomyocytes Without Use of Cardiac Stem Cells*

Adult mammalian hearts respond to injury with scar formation and not with cardiomyocyte proliferation, the cellular basis of regeneration. Although cardiogenic progenitor cells may maintain myocardial turnover, they do not give rise to a robust regenerative response. The heart, however, has dormant regenerative capacities that can be reawakened. Extracellular periostin that is abundant in the developing fetal heart (and in injured skeletal muscle) but scarce in adult hearts induces reentry of differentiated mammalian cardiomyocytes into the cell cycle. Periostin stimulates mononucleated cardiomyocytes to go through the full mitotic cell cycle by activating αV, β1, β3, and β5 integrins located in the cardiomyocyte cell membrane. A sponge-like patch soaked with periostin induced cardiomyocyte proliferation and improved heart function when placed over the site of cardiac injury in rats (Kühn et al. 2007). Similar results were seen in larger animals, and periostin is now in preclinical development at Children's Hospital (Boston, MA) for future application in human patients with heart failure. Cardiomyocytes can also be induced to proliferate and regenerate by controlling the underlying molecular mechanism for this process that involves the growth factor neuregulin1 (NRG1) and its tyrosine kinase receptor, ErbB4 (Bersell et al. 2009). NRG1 induces mononucleated, but not binucleated, cardiomyocytes to divide. In vivo, genetic inactivation of ErbB4 reduces cardiomyocyte proliferation, whereas increasing ErbB4 expression enhances it. Injecting NRG1 in adult mice induces cardiomyocyte cell-cycle activity and promotes myocardial regeneration, leading to improved function after myocardial infarction. Undifferentiated progenitor cells do not contribute to NRG1-induced cardiomyocyte proliferation. Thus, increasing the activity of the NRG1/ErbB4 signaling pathway may provide a molecular strategy to promote myocardial regeneration without need for cardiac stem cells.

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# Chapter 8
# Gene Therapy for Cardiovascular Disorders

## Introduction

Gene therapy is defined as the transfer of defined genetic material to specific target cells of a patient for the ultimate purpose of preventing or altering a particular disease state. It has three components; (1) identification of the gene mutated in the disease and to obtain a healthy copy of that gene; (2) carrier or delivery vehicle called vectors to deliver the healthy gene to a patient's cells; and (3) additional DNA elements that turn on the healthy gene in the right cells and at the right levels (Jain 1998). Gene therapy usually involves in situ production of therapeutic proteins but some approaches require suppression of gene expression to achieve therapeutic effects. Applications of gene therapy would be narrow if confined only to transfer of defined genetic material to specific target cells using vectors, which are usually viral but several nonviral vectors are used as well. Genes and DNA can be introduced without the use of vectors and various techniques are being used to modify the function of genes in vivo without gene transfer, for example, gene repair. Gene medicines may modify the effects of genes. Genetically modified cells can deliver therapeutic proteins. Gene therapy can now be combined with antisense and RNA interference (RNAi). A special report on gene therapy covers this topic in detail (Jain 2011b).

Advances in molecular pathophysiology of cardiovascular diseases have brought gene therapy within the realm of possibility as a novel approach to treatment of these diseases. Heart disease is often the end result of inherited genetic defects, which may potentially be treatable using a gene-transfer approach. It is hoped that gene therapy will be less expensive and affordable because the techniques involved are simpler than those involved in cardiac bypass surgery, heart transplantation, and stent implantation. Gene therapy would be a more physiologic approach to deliver vasoprotective molecules to the site of vascular lesion. With over 40 clinical human trials, either completed or currently enrolling, cardiovascular gene therapy has proven to be safe, and initial results suggest its efficacy. Some of the cardiovascular disorders for which gene therapy is being considered currently are shown in Table 8.1.

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**Table 8.1** Cardiovascular disorders for which gene therapy is being considered

| |
|---|
| *Genetic cardiovascular disorders* |
| Familial hypercholesterolemia |
| Hypertension |
| Familial hypertrophic cardiomyopathy |
| Long Q-T syndrome |
| Genetic factors in coronary heart disease |
| *Acquired cardiovascular disease* |
| Coronary artery disease/angina pectoris |
| Ischemic heart disease/myocardial infarction |
| Congestive heart failure and cardiomyopathy |
| Pulmonary hypertension |
| Cardiac conduction disturbances |
| *Gene therapy and cardiac transplantation* |
| *Peripheral arterial disease* |
| Prevention of atherosclerosis |
| Prevention of restenosis after angioplasty |
| Maintaining the patency of vascular grafts |
| Intermittent claudication |



## Techniques of Gene Transfer to the Cardiovascular System

Once an appropriate molecular target has been identified, the cellular target of the proposed genetic manipulation must be chosen. Although this usually means the cell type most intimately involved in the disease process, it can also be a neighboring cell type or an organ at a distance from the site of lesion. Local delivery of genes to the sites of disease is a salient feature of cardiovascular gene therapy.

Various target genes and methods of gene transfer have been considered and investigated. Special techniques have been developed to target certain areas within the cardiovascular system and these include direct injection, percutaneous transluminal approaches and special catheters. Gene transfer for cardiovascular diseases can be done ex vivo or in vivo. Ex vivo modification of endothelial and vascular smooth muscle cells followed by implantation of the cells seeded on prosthetic graft material or onto luminal surface of native vessels has been shown to be feasible and local or systemic delivery of a transgene can be accomplished. However, gene transfer is cumbersome and inefficient by this approach. In vivo gene transfer is preferred for the cardiovascular system.

### *Direct Plasmid Injection into the Myocardium*

A gene can be transferred to the myocardium by direct injection of a plasmid (a circular, double-stranded unit of DNA). A special plasmid that could transfer a gene for vascular endothelial growth factor (VEGF), which promotes angiogenesis,

has been injected into patients with coronary artery disease. A placebo-controlled clinical trial has assessed the safety and effects of combined treatment with the plasmid VEGF-A165 and granulocyte-colony stimulating factor (G-CSF) mobilization of bone marrow stem cells in patients with severe chronic ischemic heart disease (Ripa et al. 2006). It was concluded that intramyocardial VEGF-A165 gene transfer followed by bone marrow stem cell mobilization with G-CSF was safe. However, a significant increase in circulating stem cells did not lead to improved myocardial perfusion or clinical effects suggesting a neutral effect of the treatment. To improve homing of stem cells, higher doses of VEGF-A165 and/or use of SDF-1 transfer might be considered.

## *Catheter-Based Systems for Vector Delivery*

Catheter-based systems for vector delivery are shown in Table 8.2 along with their advantages and disadvantages.

## *Ultrasound Microbubbles for Cardiovascular Gene Delivery*

Ultrasound-targeted microbubble destruction can be used to deliver adenoviral or plasmid DNA to the myocardium. This technique holds great promise in applying the rapidly expanding repertoire of gene therapies being developed for cardiac disease.

Encapsulated gas microbubbles are well known as ultrasound contrast agents for medical ultrasound imaging. They can also be used as drug/gene carriers. The microbubbles as drug/gene carriers have an average size less than that of red blood cells, that is, they are capable of penetrating even into the small blood capillaries and releasing drug and genes under the action of ultrasound field. The application of ultrasound and microbubbles to targeted drug and gene delivery has been the subject of intense experimental research. Under exposure of sufficiently high-amplitude ultrasound, these targeted microbubbles would rupture, spilling drugs or genes, which are contained in the encapsulating layer, to targeted cells or tissues. Recently, targeting ligands are attached to the surface of the microbubbles (i.e., targeted-microbubbles), which have been widely used in cardiovascular system (Liu et al. 2006). Novel targeted ultrasonic contrast agents or microbubbles have potential applications in cardiovascular gene therapy.

## *Vectors for Cardiovascular Gene Therapy*

Both viral and nonviral vectors have been used for gene transfer to the cardiovascular system. Nonviral vectors consist mainly of DNA plasmids. Retroviral vectors have limited gene transfer efficacy because of low number of proliferating cells in the cardiovascular system.

**Table 8.2** Catheter-based systems for vector delivery to the cardiovascular system

| Catheter | Advantages | Disadvantages/limitations |
|---|---|---|
| *Gene transfer to the vessel wall* | | |
| Double balloon | Prolonged contact of vector with vessel wall | Occlusion of the vessel |
| | Minimal systemic spread of the vector | Vector escape via side branches |
| | Enables withdrawal of vector after gene transfer | Transduction efficacy low |
| Porous balloon | Transfer to deeper layers of vessel wall<br>Short occlusion time<br>Enables simultaneous PTCA and gene transfer | Damage to the vessel wall from high pressure delivery |
| Gel-coated balloon | Short vessel occlusion time<br>Enables simultaneous PTCA and gene transfer | Low gene transfer efficacy |
| Dispatch Catheter | Allows distal blood flow<br>Prolonged contact of vector with vessel wall<br>Efficient gene delivery<br>Minimal systemic spread of the vector | Vector escape via side branches |
| Angioplasty balloon | Transfer to deeper layers of vessel wall<br>Short occlusion time<br>Enables simultaneous PTCA and gene transfer<br>Minimal systemic spread of the vector | Damage to the vessel wall from multiple injections |
| *Gene transfer to the myocardium* | | |
| NOGA | Enables percutaneous access to myocardial muscle<br>Gene transfer to viable ischemic myocardium<br>High gene transfer efficacy<br>Minimal systemic spread of the vector | High cost<br>Difficult and time consuming |
| *Gene transfer to vessel wall as well as the myocardium* | | |
| Micro-Infusion Catheter | Percutaneous procedure<br>Direct delivery to vessel wall and myocardium<br>Perivascular delivery enables gene to be retained at treatment site and not lost in blood circulation | Approved only for delivery into the vessel wall or perivascular area<br>Research in progress for vessels 2–8 mm in diameter |



## Adenoviral Vectors for Cardiovascular Diseases

Adenoviral vectors are considered to be the most effective agents for gene transfer for vascular disease. Gene transfer of vasoactive molecules is an effective method of vascular protection. Clinical trials in humans followed the success of intracoronary injection of adenoviral vectors encoding VEGF in pigs with coronary occlusion. Revascularization takes place by a process of angiogenesis. Such vectors have

also been injected directly into myocardium during coronary bypass operation patients. In phase I/II clinical trials, nonsurgical injection of adenoviral vectors carrying FGF genes was carried out via an intra-arterial catheter into coronary arteries of patients with exertional angina pectoris.

However, transfection efficiency achievable with adenoviral vectors is attenuated in the presence of atherosclerotic lesions. The routes of delivery have also been investigated. Perfusion channel designs are being studied to obviate the lengthy period of arterial occlusion necessary for obtaining abundant gene expression. In vivo gene transfer has been done with a perforated balloon catheter but the number of cells that can be transduced is limited. Transcatheter route still remains an effective route for delivery of recombinant DNA sequences into the coronary artery wall. However, the state of recipient cells is a key determinant of the long-term transgene expression following direct gene transfer into the coronary vasculature.

Intracoronary delivery of recombinant adenoviruses encoding angiogenic proteins that contain signal peptides (FGF-2FGF-4 and FGF-5) can ameliorate myocardial ischemia. The presence of the signal peptide is an important element in the favorable effects that transgene expression has on regional flow and function in an animal model of myocardial ischemia (Gao et al. 2005). These authors have reported that 2 weeks following gene transfer of FGF-2LI containing the signal peptide, regional abnormalities in stress-induced function blood flow were improved. In the absence of the signal peptide, perfusion was not improved, and function was improved to a lesser degree than with FGF-2LI containing the signal peptide. These studies indicate that the presence of a signal peptide increases the efficacy of treatment and may reduce the required recombinant adenovirus dose for a given effect, and thereby provide an important safety margin for clinical application.

### Plasmid DNA-Based Delivery in Cardiovascular Disorders

The direct injection of naked DNA into myocardium is simple and has low toxicity but the efficiency is low. To achieve stable expression, DNA must be integrated into the host genome. DNA is usually complexed with liposomes.

Cationic liposome preparations are efficient vectors that increase gene expression in arteries compared to naked DNA. The improvement in gene expression, together with relative safety, suggests that these vectors may be appropriate gene therapy in cardiovascular diseases using catheter-based gene delivery.

Water-soluble lipopolymer, which consists of polyethylenimine and cholesterol, is an efficient carrier for local gene transfection to myocardium, and useful in in vivo gene therapy.

### Intravenous rAAV Vectors for Targeted Delivery to the Heart

Long-term gene expression in the heart has been achieved previously but it was with direct injection into the heart muscle and it was inefficient. It is now possible to deliver a much lower dose of the vector into a vein like any other drug, and the

corrective gene is delivered to the heart. Recombinant adeno-associated virus (rAAV)-mediated gene delivery has emerged as a realistic method for the treatment of heart disease due to inherited genetic defects. A study monitored by ECG tracings has demonstrated physiological disease correction by AAV9 gene transfer in a mouse model of Pompe disease, a form of muscular dystrophy that damages the heart (Pacak et al. 2006). Intravenous delivery of the AAV9 preferentially transduced cardiac tissue in nonhuman primates and holds promise for long-lasting treatments of hereditary diseases of the heart. Studies in patients with Pompe disease are planned.

#### Molecular Cardiac Surgery with Recirculating Delivery of AAV Vectors

A novel technique termed molecular cardiac surgery with recirculating delivery (MCARD) enables closed recirculation of vector genomes in the cardiac circulation using cardiopulmonary bypass (white et al. 2011). This technique was shown to be highly efficient in isolating the heart from the systemic circulation in vivo. Using MCARD, the investigators in this study compared the relative efficacy of single-stranded (ss) AAV6 (ssAAV9) and self-complimentary (sc)AAV6-encoding enhanced green fluorescent protein, driven by the constitutive cytomegalovirus promoter to transduce the ovine myocardium in situ. MCARD enabled delivery of up to 48 green fluorescent protein genome copies per cell globally in the sheep left ventricular (LV) myocardium. It was demonstrated that scAAV6-mediated MCARD delivery results in global, cardiac-specific LV gene expression in the ovine heart and provides for considerably more robust and cardiac-specific gene delivery than other available delivery techniques such as intramuscular injection or intracoronary injection. This technique has the potential for translation into clinical application for heart failure gene therapy.

### *Hypoxia-Regulated Gene Therapy for Myocardial Ischemia*

Early intervention with a novel gene therapy, combining a therapeutic gene with a genetic biosensor that recognizes and responds to the oxygen deprivation following ischemia from coronary artery disease, might effectively prevent the organ damage commonly suffered by heart attack victims. As soon as the oxygen declines, the sensor turns on the therapeutic gene, thereby protecting the heart. Application of this regulatable system using an endogenous physiological stimulus for expression of a therapeutic gene may be a feasible strategy for protecting tissues at risk of ischemia/reperfusion injury. In addition to its potential for patients with heart disease, the strategy might also prove useful for any condition in which tissues are susceptible to loss of blood supply, including stroke, shock, trauma, and sepsis.

The therapeutic gene construct containing both DNA sequences that can detect oxygen deficiency and a therapeutic human gene – heme-oxygenase 1 – has been shown to protect cells. It was inserted into heart, liver, and skeletal muscle of rats

by an AAV vector prior to induction of ischemia and oxygen deprivation with no evidence of damage. The results of this study will be verified in a large animal model. If the findings hold, the therapy might be ready to enter a phase I clinical trial in human patients.

## *Angiogenesis and Gene Therapy of Ischemic Disorders*

Angiogenesis (formation of blood vessels) is fundamental to reproduction, development, and repair. During human embryonal growth, vessels develop to deliver adequate nourishment and oxygen from the maternal circulation. Angioblasts of extraembryonic mesoderm give rise to primitive vascular channels and angiogenesis originates from these structures. In infants, angiogenesis is proportional to the proliferation of the tissue in which it takes place and declines in childhood. In healthy adults, the turnover of endothelial cells is extremely low and angiogenesis essentially does not take place.

Angiogenesis is a complex multistep process involving extensive interplay between cells, soluble factors, and extracellular matrix components. Several angiogenic peptides have been discovered. Some stimulate vascular endothelial cells to proliferate whereas others act indirectly by mobilizing host cells to release endothelial growth factors. The activity of these angiogenic factors is counteracted by endogenous inhibitors of angiogenesis. Angiogenesis can be triggered by various humoral stimuli and occurs in some diseases such as retinopathy of prematurity and hemangiomas. Endothelial proliferation within atherosclerotic plaques of coronary arteries can lead to hemorrhage and initiate a heart attack. Angiogenesis also occurs during vascularization of tumors. Angiogenesis is a normal component of wound healing, and angiogenic factors can improve the healing of chronic wounds.

Angiogenesis is a target for ischemic diseases with an intention to stimulate it whereas the aim is to suppress it in cancer. Advances in viral and nonviral vector technology including cell-based gene transfer have continued to improve transgene transmission and expression efficiency. An alternative strategy to the use of transgenes encoding angiogenic growth factors is therapy based on transcription factors such as hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$) that regulate the expression of multiple angiogenic genes. Cardiovascular gene therapy with VEGF has yielded improved perfusion and reduced ischemia in preclinical models of ischemic heart disease (IHD). Several preclinical studies and phase I/II clinical trials have shown the safety and therapeutic potential of gene therapy in the treatment of IHD, peripheral arterial disease (PAD), restenosis, and ischemic and diabetic neuropathy, pointing to the need for phase III clinical trials.

Unregulated VEGF-mediated angiogenesis has the potential to promote tumor growth, accelerate diabetic proliferative retinopathy, and promote rupture of atherosclerotic plaque. To be safe and effective, gene therapy with VEGF must be regulated. To limit the risk of pathological angiogenesis, hypoxia-inducible VEGF gene therapy systems have been devised. For example, injection of a water-soluble lipopolymer and an erythropoietin enhancer can induce expression of VEGF in

ischemic myocardium and not in the normal myocardium. Localized induction of VEGF and the low cytotoxicity of such a system support its potential as treatment for IHD.

## *Therapeutic Angiogenesis with Vascular Endothelial Growth Factor Therapy*

Therapeutic angiogenesis can be achieved with recombinant vascular endothelial growth factor proteins or gene encoding for the proteins. The greatest interest and research has been concentrated on basic Fibroblast Growth Factor (FGF1 and FGF2) and Vascular Endothelial Growth Factor A (VEGF-A165 and VEGF-A121). Several small clinical phase I–II safety and efficacy trials with recombinant VEGF proteins or gene encoding for the proteins have demonstrated that these treatment regimes seem to be safe and the results have been encouraging. However, two large double-blind randomized placebo-controlled studies with intracoronary infusions of the recombinant proteins FGF2 and VEGF-A165 could not detect any clinical effect (Kastrup 2003). Therapeutic angiogenesis is still a promising treatment in patients with coronary artery disease. However, more research including large-scale clinical trials is needed before deciding whether the vascular endothelial growth factor therapy either as a gene or a recombinant slow-release protein formulation therapy can be offered to patients with severe coronary artery disease, which cannot be treated with conventional revascularization.

A hypoxia-inducible VEGF expression vector was introduced into mesenchymal stem cells (HI-VEGF-MSCs) using a nonviral delivery method, which were then used for the treatment of ischemic myocardial injury in rats (Kim et al. 2011). VEGF expression from HI-VEGF-MSCs was significantly increased by hypoxia in vitro as compared to normoxia. Similarly, in vivo administration of HI-VEGF-MSCs induced ischemia-responsive VEGF production, leading to a significant increase in myocardial neovascularization after MI. As compared to unmodified MSCs, HI-VEGF-MSCs were retained in infarcted myocardium in greater numbers and remarkably reduced the number of apoptotic cells in the infarcted area. These investigators showed that transplantation of HI-VEGF-MSCs resulted in a substantial attenuation of left ventricular remodeling in rat MI. This study demonstrates that cell-based gene therapy using genetically engineered MSCs to express VEGF in response to hypoxic stress can be a promising therapeutic strategy for the treatment of ischemic heart disease.

## *Gene Painting for Delivery of Targeted Gene Therapy to the Heart*

In experiments with pigs, scientists at Johns Hopkins (Baltimore, MD) have successfully used a technique called “gene painting” to target gene therapy to a specific region of the heart and change the heart’s rhythm. The technique could help in the

development and delivery of future gene therapies for atrial fibrillation, a common disorder in which the electrical signaling that triggers the heartbeat goes awry. A controlled study evaluated whether gene therapy using the gene HERG-G628S that helps regulate the heartbeat, could effectively alter the heartbeat in pigs with an irregular heart rhythm. The gene was contained in a plastic, gel-like substance that was painted onto the surface of the right atrium of the heart. The gel also contained a dye so that its spread could be tracked inside the organ. After 3 weeks, the heartbeat had returned to normal, and the dye had penetrated only the atria.

## *Gene Delivery to Vascular Endothelium*

In general, viral methods have been shown to be very effective at delivering genes to cardiovascular endothelium. The immunogenicity and pathogenicity associated with viral vectors have led increased efforts to seek alternative means of "ferrying" therapeutic genes to endothelium or to decrease the shortcomings of viral vectors. Various nonviral methods such as chemical vectors can deliver DNA to cells and may represent a robust and versatile technology to "ferry" therapeutic genes to vascular endothelium in order to modify the endothelial dysfunction associated with many cardiovascular diseases (Theoharis et al. 2007).

## *Targeted Plasmid DNA Delivery to the Cardiovascular System with Nanoparticles*

Targeting gene therapy to the cardiovascular system is a challenge. Biodegradable polymeric superparamagnetic nanoparticle formulations have been formulated using a modified emulsification-solvent evaporation methodology with both the incorporation of oleate-coated iron oxide and a polyethylenimine oleate ion-pair surface modification for DNA binding (Chorny et al. 2007). The DNA was in the form of a plasmid, a circular molecule that carried a gene that coded for a growth-inhibiting protein adiponectin.

Magnetically driven nanoparticle-mediated gene transfer was studied using a green fluorescent protein reporter plasmid in cultured arterial smooth muscle cells and endothelial cells. Nanoparticle-DNA internalization and trafficking were examined by confocal microscopy. Cell growth inhibition after Nanoparticle-mediated adiponectin plasmid transfection was studied as an example of a therapeutic end point. Nanoparticle-DNA complexes protected DNA from degradation and efficiently transfected quiescent cells under both low and high serum conditions after a 15 min exposure to a magnetic field. There was negligible transfection with nanoparticle in the absence of a magnetic field. Larger sized nanoparticles (375 nm diameter) exhibited higher transfection rates compared with 185 nm- and 240 nm-sized nanoparticles. Internalized larger sized nanoparticles escaped lysosomal

localization and released DNA in the perinuclear zone. Adiponectin plasmid DNA delivery using nanoparticles resulted in a dose-dependent growth inhibition of cultured arterial smooth muscle cells. It is concluded that magnetically driven plasmid DNA delivery can be achieved using biodegradable nanoparticles containing oleate-coated magnetite and surface modified with PEI oleate ion-pair complexes that enable DNA binding. The materials composing the nanoparticles are biodegradable, so they break down into simpler, nontoxic chemicals that can be carried away in the blood. This addresses the safety concerns of the use of nonbiodegradable nanoparticles in vivo. As a nonviral method, it avoids the unwanted immune system responses that have occurred when viruses are used to deliver gene therapy.

Although the research done in cell cultures is in early stages, it may represent a new method for delivering gene therapy to benefit blood vessels damaged by arterial disease. Such nanoparticles could be magnetically directed into stents inserted into a patient's partially blocked vessels to improve blood flow. Delivering antigrowth genes to stents could help prevent restenosis. The magnetically driven delivery system also may find broader use as a vehicle for delivering drugs, genes, or cells to a target organ. After preloading genetically engineered cells with nanoparticles, researchers could use magnetic forces to direct the cells to a target organ. Furthermore, researchers might deliver nanoparticles to magnetically responsive, removable stents in sites other than blood vessels, such as airways or parts of the gastrointestinal tract. After the nanoparticles have delivered a sufficient number of genes, cells, or other agents to have a long-lasting benefit, the stent could be removed.

### eNOS Gene Therapy for Restenosis

Endogenous NO in the vasculature is vasoprotective by inhibiting platelet and leukocyte adhesion, inhibiting smooth muscle cell (SMC) proliferation and migration, and promoting endothelial survival and proliferation. At sites of vascular injury following angioplasty, the endothelium is disrupted and NO synthesis is impaired. Hence, augmenting local NO synthesis through eNOS gene transfer may help arrest the proliferative response to vascular injury. Several experimental studies have shown that delivery of eNOS gene to balloon-injured rat carotid arteries using viral as well as nonviral vectors results in reduction in neointimal formation.

The first multicenter, prospective, single-blind, dose escalation study was conducted to obtain safety and tolerability information of the iNOS-lipoplex (CAR-MP583) gene therapy for reducing restenosis following PCI (von der Leyen et al. 2011). Local coronary intramural CAR-MP583 delivery was achieved using the Infiltrator balloon catheter. There were no complications related to local application of CAR-MP583. In one patient, PCI procedure-related transient vessel occlusion occurred with consecutive troponin elevation. There were no signs of inflammatory responses, hepatic toxicity, or renal toxicity. No dose relationship was seen with regard to adverse events across the dose groups. It was concluded that coronary intramural lipoplex-enhanced iNOS gene therapy during PCI is feasible and appears to be safe. These initial clinical results are encouraging to support further clinical research, particularly in conjunction with new local drug delivery technologies.

# Gene Therapy for Genetic Cardiovascular Disorders

## *Genetic Disorders Predisposing to Atherosclerosis*

Atherosclerosis is no longer considered to be a degenerative disease or a consequence of aging. Rather, it is a chronic inflammatory condition, which can be converted to an acute condition by induction of plaque rupture, which leads to thrombosis. The basic mechanism is lipoprotein transport into the vessel wall, which initiates the process. Atherosclerosis is induced in several animal species by mutations that involve the LDL (low density lipoprotein) receptor gene, providing strong evidence that elevations in LDL are sufficient to induce all the components of the atherosclerotic reaction. The prevention and treatment strategies for atherosclerosis are directed mainly at lowering LDL levels or raising HDL (high density lipoprotein) levels. Identification of major genes that control the formation of biologically active oxidized lipids or control the intensity of response to these lipids will enable development of strategies to favorably modify the functions controlled by these genes.

HDL contains apolipoprotein (apo) A-1, the overexpression of which leads to elevated concentrations of HDL and reduced risk of atherosclerosis. Gene transfer methods are available that can be used to overexpress apo A-1. This overexpression of this gene may mitigate the deleterious effects of other genetic factors in atherosclerosis.

Apolipoprotein is the major protein component of both LDL and lipoprotein (Lp) a. It is an independent risk factor for atherosclerosis. No therapies are available currently for lowering plasma Lp(a) levels. Availability of apoB is an important determinant of Lp(a) concentrations and reduction of apoB plasma concentration may provide a novel approach for lowering Lp(a) levels. Adenovirus-mediated gene transfer of cytidine deaminase apoBEC-1 has been shown to lower Lp(a) in transgenic mice.

The use of retroviral transduction of hematopoietic stem cells (HSCs) for treatment of patients with atherosclerosis still remains a long-term goal. However, the recent development of retroviral vectors capable of directing expression to specific cell types within the lesion will allow more targeted therapeutic strategies to be devised. In addition, these vectors will provide powerful experimental tools to further our understanding of the pathogenesis of the disease. Retroviral and lentiviral vectors encoding siRNAs clearly show their potential for gene therapy for many human diseases. By using these vectors to transduce HSCs they should also prove to be of great therapeutic value for inhibiting the expression of pro-atherogenic gene expression by cells within atherosclerotic lesions.

## *Gene Therapy of Familial Hypercholesterolemia*

Even with the most effective of the currently available statins, a number of patients with familial hypercholesterolemia (FH) are still failing to reach treatment guidelines for the control of hypercholesterolemia. The success of orthotopic liver

transplantation in normalizing serum LDL of patients with homozygous FH suggests a strategy of gene therapy directed to the liver.

Gene therapy by introduction of functional LDL receptors genes would be expected to be beneficial for these patients. The gene for LDL receptor has been localized to chromosome 19p13.1–p13.3 and over 200 mutations of the LDL-R gene have been reported. The Watanabe Heritable Hyperlipidemic (WHHL) rabbit has been used as a model for gene therapy studies. Liver tissue was removed from the rabbit, hepatocytes were cultured and transfected by retroviruses with LDL receptor gene. There was reduction in cholesterol of 30–40%, which persisted for several months. This ex vivo approach has been used to treat human patients in a clinical trial with demonstration of significant and prolonged reduction in LDL cholesterol. Although the feasibility of ex vivo liver-directed gene therapy was shown, variations in the metabolic response in the patients preclude further expansion of the trial without modifications.

Transplantation of hepatocytes, whether genetically modified or not, has become an alternative to orthotopic liver transplantation for the treatment of patients with metabolic diseases. However, more than a decade after the first clinical trial of ex vivo gene therapy to treat patients with FH, there are still a number of impediments to these approaches. Numerous animal models are still being developed on the one hand to improve hepatocyte integration within hepatic parenchyma and function, and on the other hand to develop vectors that drive long-term transgene expression in situ. These include large animal models such as nonhuman primates, which have recently led to significant progress in hepatocyte transplantation. Simultaneous development of lentiviral vectors from different lentivirus species has permitted the transfer of genes into mitotically quiescent primary cells including differentiated hepatocytes. Particularly third generation vectors derived from HIV-1 lentivirus are the most widely used and have significantly improved the safety and efficiency of these vectors. Given the shortage of organs and problems related to immunosuppression on one hand, and recent progresses in hepatocyte transduction and transplantation on the other hand, ex vivo approach is becoming a real alternative to allogeneic hepatocyte transplantation (Nguyen et al. 2009).

Nitric oxide synthase (NOS) gene therapy rapidly ameliorates several biomarkers of atherosclerosis in the cholesterol-fed rabbit. A rabbit model of human FH has been used to develop an in vivo approach to gene therapy based on recombinant adenoviruses. Recombinant, replication defective adenoviruses expressing the lacZ gene under the control of different promoters were infused into the portal circulation of New Zealand White rabbits. The development of neutralizing antibodies to the recombinant adenovirus markedly diminishes the effectiveness of the second dose. An alternative strategy is ectopic expression in the liver of very low-density lipoprotein (VLDL) receptor, which is homologous to the LDL receptor but has a different pattern of expression. Infusion of recombinant adenoviruses containing VLDL receptor gene corrects the dyslipidemia in the FH mouse and circumvented immune responses to the transgene, leading to a more prolonged metabolic correction.

Helper-dependent adenovirus (HD-Ad) gene transfer as treatment of LDLR−/− mice leads to long-term overexpression of apoA-I, retards atherosclerosis progression, and remodels the lesions to a more stable-appearing phenotype.

HD-Ad-mediated transfer of apoA-I may be a useful clinical approach for protecting against atherosclerosis progression and stabilizing atherosclerotic lesions associated with dyslipidemia in human patients, but it has not been tested clinically.

Transferrin-facilitated intravenous transfer of a cationic liposome rabbit LDLR cDNA complex has been shown to alleviate hypercholesterolemia in Watanabe Heritable Hyperlipidemic Rabbits, an animal model of FH. Intravenous treatment decreases plasma total as well as LDL cholesterol levels in a dose-dependent manner, correlating with an increased level of LDLR mRNA transcripts in leukocytes. This could serve as an important adjunct therapy for the treatment of FH.

Permanent phenotypic correction with single administration of a gene therapeutic vector still remains a goal that needs to be achieved. The first ex vivo clinical trial of gene therapy in FH was conducted nearly 18 years ago. Patients who had inherited LDLR gene mutations were subjected to an aggressive surgical intervention involving partial hepatectomy to obtain the patient's own hepatocytes for ex vivo gene transfer with a replication deficient LDLR-retroviral vector. After successful re-infusion of transduced cells through a catheter placed in the inferior mesenteric vein at the time of liver resection, only low-level expression of the transferred LDLR gene was observed in the five patients enrolled in the trial. In contrast, full reversal of hypercholesterolemia was later demonstrated in in vivo preclinical studies using LDLR-adenovirus-mediated gene transfer. However, the high efficiency of cell division independent gene transfer by adenovirus vectors is limited by their short-term persistence and cytotoxicity of these highly immunogenic viruses. Novel long-term persisting vectors derived from adeno-associated viruses and lentiviruses, are now available and investigations are underway to determine their safety and efficiency in preparation for clinical application for a variety of diseases. Several novel nonviral-based therapies have also been developed recently to lower LDL-C serum levels in FH patients (Al-Allaf et al. 2011).

The following minimal criteria are proposed for conduct and evaluation of further investigations of gene therapy in hypercholesterolemia:

- Animal experiments should provide clear-cut evidence for the persistent activity of the exogenously transferred LDL receptor gene in mediating the removal of LDL cholesterol.
- The observed reduction in LDL should be shown to result from increased activity of LDL receptors.
- Increased receptor activity should be shown to result from expression of the exogenously derived gene rather than from activation of the patient's own receptors in response to surgical or other manipulation of the liver.

## *Apolipoprotein E Deficiency*

ApoE deficiency has also been shown to cause severe hyperlipidemia and atherosclerosis in humans and gene-targeted mice. Although most of ApoE in plasma is of hepatic origin, it is also synthesized by a variety of other cell types, including

macrophages. ApoE-deficient mice given transplants of normal bone marrow show virtually complete protection from diet-induced atherosclerosis. Overexpression of ApoE may provide effective gene therapy for hyperlipidemia and atherosclerosis in conditions other than ApoE deficiency. Bone marrow has the potential to serve as a vehicle for gene therapy to treat hyperlipidemia and atherosclerosis. Intravenous injection of adenoviral vectors containing the apoE gene into ApoE-deficient mice has been shown to normalize ApoE levels and lead to a marked reduction in atherosclerosis.

## *Hypertension*

Several genes are involved in the regulation of blood pressure and it is more difficult to identify them than is the case with single gene disorders. Transgenic rats created by injection of renin and angiotensinogen genes into the germline manifest hypertension. In mice carrying the angiotensinogen gene, the hypertension seems to be caused by an overexpression of this gene in the liver and the brain. A BP1 gene, which has an important effect on blood pressure, is located on the rat chromosome 10 and is closely linked to the gene encoding angiotensin-converting enzyme. There is a significant but weaker linkage to BP2 locus on chromosome 19. Based on the observation that transgenic mice expressing human kallikrein develop sustained hypotension, significant reduction of blood pressure has been demonstrated by injecting a kallikrein gene construct into the skeletal muscle of spontaneously hypertensive rats. Kallikrein is a peptide that is involved in the production of kinin, a potent vasodilator. Gene therapy with human tissue kallikrein may have potential as a treatment for hypertension and associated insulin resistance. Experimental studies suggest that the beneficial effects of human tissue kallikrein on these parameters are associated with changes in endothelin-1, endothelin-A receptor, and angiotensin II receptor type 1 expression. A study in rats indicates that rAAV-mediated human tissue kallikrein gene delivery is a potentially safe method for the long-term treatment of hypertension and could be applied in the salt-sensitive population to prevent the occurrence of hypertension (Yan et al. 2008).

Intravenous transfer of human atrial natriuretic peptide (ANP) gene has demonstrated similar results. Human atrial natriuretic peptide gene delivery also significantly attenuates salt-induced aortic hypertrophy, as evidenced by reduced thickness of the aortic wall, and reduces the mortality rate caused by cerebrovascular disorders. Successful application of this technology may have potential value in treating individuals with a high risk of stroke. Retroviral constructs designed to produce a protein that is antisense to a portion of the angiotensin-type 1 receptor produce a more sustained reduction of blood pressure in spontaneously hypertensive rats than losartan potassium. One limitation of ANP gene therapy is the need for long-term ANP gene expression and its control. A helper-dependent adenoviral vector carrying the mifepristone (Mfp)-inducible gene-regulatory system has been described to control in vivo ANP expression in a mouse model of hypertension (Schillinger et al. 2005).

Physiological effects of ANP, including decreased systolic blood pressure, increased urinary cGMP output, and decreases in heart weight as a percentage of body weight were also under the control of Mfp. Given these capabilities, this vector represents a paradigm for the gene therapy of hypertension.

Hepatocyte growth factor (HGF) has close relationships with hypertension, arteriosclerosis, and heart failure. HGF enhances renal regeneration and suppresses the progression of hypertension. Intramuscular electroporation of the therapeutic gene is a simple and economic method as a remedy for hypertension with low toxic compared to systemic administration of the purified proteins or peptides (Komamura et al. 2008).

### *Genetic Factors for Myocardial Infarction*

Mutations of the gene encoding angiotensin-converting enzyme (ACE) have been associated with increased risk of myocardial infarction. The main function of ACE is to convert angiotensin I into angiotensin II, which exerts its cellular actions through the angiotensin II AT1 receptor subtype AGT1 R. Analysis of DNA from patients with myocardial infarction shows a significant interaction between ACE and AGT1 R gene polymorphism. The findings of this study suggest that those carrying the ACE DD genotype and AGT1 R C allele are at higher risk for myocardial infarction. These findings have clinical implications for the prevention and treatment of coronary heart disease. Gene therapy could potentially cure these patients by introduction of a dominant-negative mutation.

## Gene Therapy for Acquired Cardiovascular Diseases

This category includes the most common heart diseases such as coronary artery disease (CAD), angina pectoris, myocardial infarction (MI), and congestive heart failure (CHF). Role of gene therapy in the treatment of these disorders will be discussed in this section. Gene therapy strategies could reduce the incidence of heart disease by correcting the gene defects responsible for insulin-dependent diabetes mellitus, hyperlipidemia, and hypertension.

### *Coronary Artery Disease with Angina Pectoris*

The use of gene therapy has been explored in improving the management of vascular occlusive disease. Angiogenic gene therapy for stable angina is aimed at promoting new blood vessel formation in the heart, thus providing enhanced cardiac perfusion, symptom relief, increased exercise capacity, improved quality of life, and reduced risk of coronary events.

### Ad5FGF-4

Ad5FGF-4 (alferminogene tadenovec) is a replication-deficient serotype 5 adenovirus encoding the gene for fibroblast growth factor-4 (FGF-4) driven by a cytomegalovirus promoter. In preclinical studies using a pig model of myocardial ischemia, a single intracoronary infusion of Ad5FGF-4 was shown to improve cardiac contractile function and regional blood flow in the ischemic region during stress. Histological evidence of capillary angiogenesis was observed. FGF-4 gene expression was detected in the heart but not at extracardiac sites.

Placebo-controlled trials in humans with chronic stable angina indicate that Ad5FGF-4 increases treadmill exercise duration and improves stress-related ischemia. More patients receiving Ad5FGF-4 than placebo reported complete resolution of their angina and no nitroglycerin use. Ad5FGF-4 gene therapy was well tolerated. The administration procedure did not cause any adverse events, although mild, transient fever, a transient modest fall in platelet count, and a transient mild elevation in hepatic enzymes and uric acid levels occurred in a few patients. This adverse event profile concurs with other adenoviral gene trials. There was no evidence of myocarditis, retinal neovascularization, or angioma formation.

Ad5FGF-4 is being developed as Generx™ (Cardium Therapeutics Inc) as a one-time intracoronary administration from a standard cardiac infusion catheter. Generx is in a phase III clinical trial (AWARE) to evaluate the therapeutic effects in women with recurrent angina due to coronary heart disease.

## *Gene Therapy for Improving Long-Term CABG Patency Rates*

The early success of CABG is limited by low long-term patency rates of autologous saphenous vein grafts. Because current pharmacological interventions have only limited impact on vein graft patency rates, there remains a clear clinical need for effective agents to prevent failure of vein grafts in the long term. Gene therapy in vein grafts has great potential as gene delivery can be achieved ex vivo at the time of cardiac surgery, allowing transgene expression to occur rapidly post-grafting within the acute phase of vein graft remodeling. A variety of therapeutic strategies have been tested in a range of preclinical models, but few have gone to clinical trials. Clinical translation is warranted to investigate the potential of gene therapy to improve CABG patency rates in the long term.

## *Ischemic Heart Disease with Myocardial Infarction*

One approach to prevention of recurrence or progression of ischemic heart disease is to reduce smooth muscle proliferation, which is a component of intimal proliferation and the development of atherosclerosis. Genetic modification of smooth muscle in vivo can block smooth muscle proliferation and may be beneficial in the prevention

of atherosclerosis. Platelet derived growth factor (PDGF), fibroblast growth factor (FGF), and tumor growth factor (TGF-β) are also believed to be involved in the inflammatory response in atherosclerosis. Use of antisense genes against these factors offers another possibility of prevention of cardiovascular disease.

Myocardial infarction and subsequent reperfusion lead to the activation of apoptosis, and the final destruction of the cell. An experimental approach to management of myocardial infarction is to transfer genes that stop programmed cell death or apoptosis and promote the regeneration of new cells. Inhibition of caspases, the main executioners of apoptosis, improves functional outcome after ischemia and reperfusion in animal models. Adenoviral gene transfer of the caspase inhibitor p35 leads to a significant reduction of the myocardial infarct size after ischemia and reperfusion as well significant improvement of hemodynamic variables (Bott-Flugel et al. 2005). Targeted inhibition of apoptosis in the myocardium appears to be a promising approach for ameliorating the effects of ischemia and reperfusion. Although genes to promote these functions can be transferred to cardiac muscle cells in animals using adenoviral vectors, clinical applications of this are some years away.

Another aim of gene therapy is to counteract the cellular toxic effects of blood components that are known to participate at sites of thrombosis and hemorrhage. Overexpression of heme oxygenase (HO), a stress protein that participates in defense mechanisms against such oxidative injury, has been demonstrated by transfection of HO gene into rabbit microvessel endothelial cells.

### Angiogenesis for Cardiovascular Disease

Angiogenesis represents an excellent therapeutic target for the treatment of cardiovascular disease. It is a physiological process that underlies the manner in which the body responds to impairment of blood supply to vital organs, that is, the production of new collateral vessels to overcome the ischemic insult. A large number of preclinical studies have been performed with protein, gene, and cell-based therapies in animal models of cardiac ischemia as well as models of peripheral artery disease. Various agents including growth factors have been tested for this purpose but there is no effective proven therapy. Although transplantation of mononuclear cells (MNCs) induces angiogenesis in MI, transplantation requires a large amount of bone marrow or peripheral blood cells. Retrograde transplantation of peripheral blood MNCs expressing hVEGF efficiently induces angiogenesis and improves the impaired left ventricle function in hearts of pigs with AMI (Hagikura et al. 2010). These findings indicate that angiogenic cells and gene therapy may be useful to treat ischemic heart disease.

Several gene therapy angiogenesis trials have been conducted in humans. Numerous phase I trials with either adenovirus vectors carrying an angiogenesis gene, or naked plasmid DNA vectors harboring an angiogenic gene, have demonstrated the safety of these new gene-based products. Fibroblast growth factor (FGF) has shown promise for this indication (Jacobs 2007). Several clinical trials are now advancing in patients with severe CHD including Cardium Therapeutic's phase III FGF-4 gene therapy study in woman.

CardioVascular BioTherapeutics is conducting phase II clinical trials with the FGF-1 protein, delivered by the Myostar catheter (Biologics Delivery Systems/ Cordis Corporation). In the phase I study, FGF-1 was administered by intramyocardial injections via a mini-thoracotomy. The phase II study will utilize an injection catheter to deliver the drug to essentially the same region in the heart wall, but will not require surgical intervention. The phase II trial, ACORD (Angiogenesis for the treatment of CORonary heart Disease), use of a catheter to deliver CVBT's drug candidate, CVBT-141H, permits the comparison of its benefits to a placebo control group, which was not possible when the drug was administered during a surgical mini-thoracotomy in the phase I study.

**Myocardial Repair with IGF-1 Therapy**

The biological properties of IGF-I, including inhibition of apoptosis, adaptive cardiomyocyte hypertrophy, recruitment of cardiac progenitor cells, as well as the induction of angiogenesis and enhancement of cardiac function, provide the rationale for the development of a therapy directed at myocardial repair and restoration. This biology predicts potential of Corgentin (Cardium Therapeutics Inc) to improve functional recovery and prevent ventricular dysfunction and the associated progression to congestive heart failure following myocardial infarction and reperfusion.

The safety of systemic IGF-I protein therapy has been confirmed in multiple human clinical studies for a number of medical indications. While there is abundant published scientific literature validating the multiple beneficial cardiac effects of IGF-I, systemic IGF-I protein delivery generally lacks the ability to target cardiomyocytes for effective therapy. By targeting the heart with intracoronary, DNA-coded, myocardial-directed delivery, using the proprietary methods pioneered for the Generx development program by Cardium, mdIGF-I has the potential to induce a positive biologic response. The targeted cardiomyocytes are expected to produce sustained therapeutic protein levels in the myocardium where it is needed. Over 1,000 patients have been treated with various dose levels of IGF-I protein, and 450 patients have received Generx via intracoronary administration of DNA-based myocardial delivery of the FGF-4 angiogenic growth factor. The safety and preliminary efficacy from these studies provide further support for the clinical potential of Corgentin.

In vitro preclinical development studies have confirmed published data supporting the myocardial benefits of IGF-I in cell-based assays by protecting cardiomyocytes against apoptosis, inducing adaptive cardiomyocyte hypertrophy and inducing proliferation of human coronary artery endothelial cells. Cardium's in vivo proof-of-concept pilot study in pigs, based on its coronary occlusion/reperfusion myocardial infarct model, tested intracoronary mdIGF-I administration to promote myocardial repair following a significant heart attack (myocardial infarction). This double-blind, randomized, placebo-controlled study was designed to simulate the clinical approach in which Corgentin would be administered after emergency reperfusion therapy to a heart attack patient. Following infarction, echocardiographic analysis documented recovery and restoration of ventricular function and reversal of early

left ventricular remodeling in the Corgentin-treated group, compared to placebo. Postmortem analysis of the hearts provided convincing histological evidence of the potential for post-infarct myocardial protection with this therapy. The initial clinical studies for Corgentin will be designed in an attempt to secure product registration for use in patients with acute ST-elevation myocardial infarction undergoing percutaneous coronary intervention with or without associated fibrinolysis.

## *Congestive Heart Failure*

Although advances in medical treatments have dramatically reduced the overall mortality rate due to heart disease, death due to CHF continues to rise. The present treatments for the loss or failure of cardiovascular function include organ transplantation, surgical reconstruction, mechanical or synthetic devices, or the administration of metabolic products. Although routinely used, these treatments are not without constraints and complications. Ideal and effective therapy, particularly for end-stage CHF, has been elusive. The varied causes of heart failure include abnormalities of ion handling, cellular signaling, neurohormonal control, and apoptosis, all of which are potentially amenable to genetic manipulation.

### Rationale of Gene Therapy in CHF

The central clinical problem in CHF is a lack of therapies to target the underlying molecular defects that lead to chronic ventricular dysfunction. Gene therapy holds the promise of retarding the progression, preventing, and perhaps reversing heart failure. Substantial evidence points to a final common pathway in failing myocardium, including distinct changes in intracellular. Alterations in intracellular calcium signaling play a crucial role in the pathophysiology of heart failure, and in recent years, somatic gene transfer has been identified as an important tool to help understand the relative contribution of specific calcium-handling proteins in heart failure.

An attractive strategy to address these alterations is cardiac gene therapy and several distinct approaches have been undertaken during the last decade with impressing therapeutic benefit, at least in animal CHF models. The present focus of research is the clinical translation of cardiac gene therapy including the optimization of vectors, delivery strategies, and testing the compatibility with established pharmacologic treatment to improve the prognosis of CHF in the near future (Pleger et al. 2007).

### β-ARKct Gene Therapy

The upregulation of GPCR kinase 2 (GRK2) in failing myocardium appears to contribute to dysfunctional beta-adrenergic receptor (β-AR) signaling and cardiac function. The peptide β-ARKct, which can inhibit the activation of GRK2 and improve β-AR signaling, has been shown in transgenic models and short-term gene

transfer experiments to rescue heart failure (HF). A study was conducted in HF rats to evaluate long-term β-ARKct expression with the use of stable myocardial gene delivery using AAV6 vector (Rengo et al. 2009). β-ARKct or green fluorescent protein as a control was delivered via AAV6-mediated direct intramyocardial injection. A group was also treated with concurrent administration of the β-blocker metoprolol. Robust and long-term transgene expression was found in the left ventricle at least 12 weeks after delivery. β-ARKct significantly improved cardiac contractility and reversed left ventricular remodeling, which was accompanied by a normalization of the neurohormonal (catecholamines and aldosterone) status of the chronic HF animals, including normalization of cardiac β-AR signaling. Addition of metoprolol neither enhanced nor decreased β-ARKct-mediated beneficial effects, although metoprolol alone prevented further deterioration of the left ventricle in spite of lack of effect in improving contractility. This study shows that long-term cardiac AAV6-β-ARKct gene therapy in HF results in sustained improvement of global cardiac function and reversal of remodeling at least in part as a result of a normalization of the neurohormonal signaling axis. In addition, β-ARKct alone improves outcomes more than a β-blocker alone, whereas both treatments are compatible.

GRK2 has now been shown to lead to permanent damage after myocardial infarction. Overproduction of GRK2 following a heart attack actually stimulates pro-death pathways in myocyctes outside of the initial zone of damage. There is an inverse link between GRK2 activity and the production of NO, a molecular messenger that protects the heart against damage caused by a sudden loss of blood. When there is more GRK2, there is less NO, and vice versa. GRK2 may be affecting NO production by inhibiting the prosurvival protein kinase Akt, which itself regulates NO. These conclusions are based on a study that used gene therapy to inhibit GRK2, and found heart muscles cells in mice were substantially protected against destruction that would otherwise occur after an induced myocardial infarction (Brinks et al. 2010). Conversely, mice engineered to express excess GRK2 had more damage than would have been expected after myocardial infarction. These findings suggest that humans experiencing a heart attack might be helped with prompt delivery of a therapeutic targeting inhibition of GRK2. While it may be years before this concept can be tested in patients experiencing myocardial infarction, anti-GRK2 gene therapy could be tested in patients with CHF much sooner. A phase I clinical trial for GRK2-targeted gene therapy is preparing to be launched, pending FDA approval.

### Intracoronary Adenovirus-Mediated Gene Therapy for CHF

The adult ventricular wall cells are usually refractory to conventional procedures for the introduction of foreign genes but recombinant replication-defective adenoviruses can mediate highly efficient gene transfer into adult ventricular myocytes.

Intracoronary adenovirus-mediated $Ca^{2+}$-binding protein S100A1 gene delivery in vivo to the post-infarcted failing rat heart normalizes myocardial contractile function (Most et al. 2004). Moreover, S100A1 gene transfer restored diminished intracellular $Ca^{2+}$ transients and restored energy supply in failing cardiomyocytes. This may be a novel therapeutic strategy for the treatment of heart failure.

### AAV-Mediated Gene Transfer for CHF

Preclinical studies combining Targeted Genetics' AAV-based gene delivery technology with Celladon Corporation's portfolio of genes, SERCA2a and phospholamban gene variants, suggested that gene therapy targeting calcium cycling within the heart can improve myocardial contractility and reverse the progression of CHF. A study conducted in a sheep model, designed to have a reduction in heart function similar to that found in patients with Class III heart failure, showed significant improvement in heart function and contractility. MYDICAR® (Celladon) targets SERCA2a, resulting in increased cardiac contractility and cardiac output. It is in clinical trials in CHF patients.

### AngioCell Gene Therapy for CHF

AngioCell™ (Angiogene) program is a pioneering recuperative approach that aims to improve the function of damaged tissue, rather than merely treat the symptoms of the disease. AngioCell is intended to restore the contractility of the heart tissue with a cell therapy treatment that transplants muscle precursor cells loaded with a gene to enhance blood vessel growth. The therapy also improves the blood flow and oxygen uptake of the damaged heart by stimulating the growth of new blood vessels in the myocardium, through a process of therapeutic angiogenesis.

Treating congestive heart failure with AngioCell begins with a small skeletal muscle biopsy taken from the patient 2 weeks before surgery. Using a specialized technology process, these cells are multiplied and genetically treated with Angiogene's proprietary gene, to increase their angiogenic potential. These Angiocells are then implanted into the same patient in the infarcted area of the heart – an autologous graft. The presence of these muscle cells greatly increases the heart's cardiac function, while the angiogenic activity allows better oxygen and nutrient supply to the grafted area, thereby increasing the beneficial effects of the implanted cells. This process is called angiomyogenesis. Because the procedure is an autologous cell transplant, there is no rejection, no immunosuppression, no risk of disease transmission and no ethical controversy. Use of skeletal muscle cells has the following advantages:

- Easy to extract, purify, and expand
- Ability to colonize scar tissue
- Demonstrates great elasticity
- Long-term survival of implanted cells

### nNOS Gene Transfer in CHF

The endogenous production of nitric oxide (NO) plays an important role in cardiovascular homeostasis through its action on the central and peripheral autonomic nervous system. Profound activation of the sympathetic nervous system is characteristic of CHF. Decreased NO production enhances carotid body (CB) chemoreceptor

activity in CHF rabbits. The CBs are a pair of small arterial chemoreceptor organs, which sense blood $Pa_{O2}$, $Pa_{CO2}$, and pH, and reflexly influence cardiopulmonary function.

The effects of neuronal NO synthase (nNOS) gene transfer on CB chemoreceptor activity has been investigated in CHF rabbits (Li et al. 2005). The nNOS protein expression and NO production are suppressed in CBs of CHF rabbits, but are increased 3 days after application of an adenovirus expressing nNOS (Ad.nNOS) to the CB. A specific nNOS inhibitor, S-methyl-L-thiocitrulline, fully inhibits the effect of Ad.nNOS on the enhanced CB activity in CHF rabbits.

### eNOS Gene Therapy for Congestive Heart Failure

Overexpression of eNOS within the endothelium reduced the extent of contractile dysfunction in transgenic mice with infarct-induced CHF (Jones et al. 2003). Survival was increased by 43% in the eNOS transgenic mice compared with non-transgenic mice. Fractional shortening and cardiac output were also significantly greater in the eNOS transgenic than in non-transgenic. Interestingly, pulmonary edema was evident only in non-transgenic mice, and no evidence of pulmonary edema was observed in the eNOS transgenic mice. Thus, targeted overexpression of the eNOS gene within the vascular endothelium in mice attenuates both cardiac and pulmonary dysfunction and dramatically improves survival during severe CHF.

## *Gene Therapy for Cardiac Arrhythmias*

### Gene Transfer for Biological Pacemakers

Genetic engineering approaches offer an opportunity to modulate cellular automaticity and regulate cardiac rhythm in a manner that could have significant therapeutic potential. It is well known that ventricular myocytes exhibit a more negative diastolic potential than do pacemaker cells, in large part because of the inward rectifying potassium current/K1, which pacemaker cells lack. Taking advantage of these intrinsic electrophysiological differences, a biological pacemaker has been developed by using adenoviral gene transfer approaches. By isolating the gene responsible for/K1 (the Kir2.1 gene), mutating it to make it a dysfunctional channel (a dominant-negative), inserting the mutated gene into an adenoviral vector, and delivering the virus to the hearts of guinea pigs, the investigators are able to successfully convert some ventricular myocytes to pacemaker cells. While issues of safety and long-term efficacy need to be further established, the results of such experiments provide proof of principle that gene transfer offers great promise for treatment of electrophysiological disorders including conduction system disease.

Action potentials are prolonged in failing cardiac cells as a result of diminished potassium currents. Delay of repolarization predisposes to fatal arrhythmias.

Exposure of these cells to AdShK, an adenovirus that overexpresses potassium channels has been shown to reverse the action potential prolongation of the failing heart. This demonstrates that viral gene transfer can modify the electrical properties of the heart cells but clinical application of this therapy will require development of a sensitive mechanism to control the level and distribution of the transgene expression. In animal studies, localized gene therapy has been successfully investigated using an ion channel mutation to treat atrial arrhythmias (Fishbein et al. 2005).

A novel gene therapy approach for atrial arrhythmias uses a clarithromycin-responsive ion channel subunit mutation, hMiRP1-Q9E, cloned into an expression plasmid (Perlstein et al. 2005). In a series of pig studies, right atrial myocardium was injected at one site with hMiRP1-Q9E plasmid DNA; a separate site in the same right atrium was injected with wild-type plasmid or was sham injected. Two weeks after, transfection intravenous clarithromycin administration resulted in a site-specific, dose-dependent prolongation of the repolarization phase of the right atrial epicardial monophasic action potential (MAP) only at the hMiRPQ9E sites, but not at sham or wild-type sites. MAP recordings before clarithromycin administration did not differ between hMiRP1-Q9E and control sites. These studies show that regional control of atrial myocardial repolarization by site-specific transfection with plasmid DNA encoding an antibiotic-responsive ion channel subunit is feasible and, because hMiRP1-Q9E-transfected sites were affected only if clarithromycin was given, provide proof of concept for a posttranslational, controllable gene therapy strategy for atrial arrhythmias.

Hyperpolarization-activated and cyclic nucleotide-gated (HCN) ion channels play a critical role in maintaining a normal, evenly paced heartbeat. These channels control the flow of sodium and potassium ions in and out of cells that regulate the electrical impulses of the heart. Overexpression of an engineered HCN construct via somatic gene transfer offers a flexible approach for fine-tuning cardiac pacing in vivo. In one study, the researchers used a recombinant adenoviral vector to deliver the gene encoding a bioengineered cell-surface protein, which mimics the action of HCN ion channels, to heart-muscle cells of pigs (Tse et al. 2006). By getting heart muscle cells to produce bioengineered HCN channels, they were able to reconstruct the SA node of the heart in pigs with implanted electronic pacemakers. The catecholamine-responsive in vivo "bioartificial node" exhibited a physiological heart rate and was capable of reliably pacing the myocardium, substantially reducing electronic pacing. The study offers positive and direct evidence in living models that bioengineered cells can replace the electronic pacemaker. HCN gene-based therapy is feasible for correcting defects in cardiac impulse generation.

### Management of Arrhythmias Due to Myoblast Transplantation

Skeletal myoblasts are an attractive cell type for transplantation because they are autologous and resistant to ischemia. However, clinical trials of myoblast transplantation in heart failure have been plagued by ventricular tachyarrhythmias and sudden cardiac death. Previous research from animal transplants showed that heart tissue regrowth produced a mix of skeletal and heart muscle. The pathogenesis of

arrhythmias following myoblast transplant is poorly understood, but may be related to the fact that skeletal muscle cells, unlike heart cells, are electrically isolated by the absence of gap junctions, or protein connections between cell membranes that allow neighboring cells to communicate with each other through the exchange of ions and other electrical signals. These arrhythmias could be terminated by nitrendipine, an L-type calcium channel blocker, but not by the Na channel blocker lidocaine (Abraham et al. 2005). In gene therapy experiments, production of connexin43 was increased by injecting a viral vector carrying the gene that codes for the gap-junction protein into the cultured cells. Genetic modification of myoblasts to express the gap junction protein connexin43 decreases arrhythmogenicity in hearts transplanted with myoblasts. Combining gene therapy to replace connexin43 along with myoblast transplants may prevent the development of potentially fatal arrhythmias in patients.

### Genetically Engineered Cells as Biological Pacemakers

Through genetic engineering techniques, HCN-containing heart muscle cells are turned into specialized pacing cells that have a faster, regular, beating pattern, creating a biological pacemaker. HCN gene family contributes to normal pacemaker function in the heart, and biological pacemakers aim to replicate and enhance this functionality when the normal process fails. Previous experiments creating HCN-based biological pacemakers in vivo found that an engineered HCN2/HCN1 chimeric channel (HCN212) resulted in significantly faster rates than HCN2, interrupted by 1–5 s pauses. To elucidate the mechanisms underlying the differences in HCN212 and HCN2 in vivo functionality as biological pacemakers, newborn rat ventricular myocytes overexpressing either HCN2 or HCN212 channels were studied (Zhao et al. 2009). The HCN2- and HCN212-overexpressing myocytes manifest similar voltage dependence, current density, and sensitivity to saturating cAMP concentrations, but HCN212 has faster activation/deactivation kinetics. Different HCN channels were expressed in heart cells. Channel parameters and the effect on spontaneous rate were measured, and the channel parameters were entered into a computer simulation to explore their contribution to the rate effect. The biological pacemaker appears to respond to the body's signals in the same manner as the heart's native pacemaker, yet it requires no batteries or replacement of electrical stimulator leads and could last a lifetime. These results illustrate the benefit of screening HCN constructs in spontaneously active myocyte cultures and may provide the basis for future optimization of HCN-based biological pacemakers for cardiac arrhythmias. In addition, the HCN-expressing heart culture system could serve as a screening assay for new pharmacological agents to regulate heart rate.

## *Gene Therapy and Heart Transplantation*

Heart transplant is now an accepted treatment for patients with end-stage cardiomyopathy. Heart transplantation, however, is limited by incomplete effectiveness, significant toxicity, and failure to prevent chronic rejection. Genetic manipulation

of the donor heart at the time of removal offers the unique opportunity to produce a therapeutic molecule within the graft itself, while minimizing systemic effects. Cytoprotective approaches including gene transfer of HO-1, endothelial nitric oxide synthase, and antisense oligodeoxynucleotides specific for nuclear factor (NF)-kappa B or intercellular adhesion molecule (ICAM)-1 reduced ischemia-reperfusion injury and delayed cardiac allograft rejection in small animals. Exogenous overexpression of immunomodulatory cytokines such as interleukin (IL)-4, IL-10 and TGF-β, as well as gene transfer of inhibitors of pro-inflammatory cytokines also delayed graft rejection. Gene transfer-based blockade of T-cell costimulatory activation with CTLA4-Ig or CD40-Ig has resulted in long-lasting graft survival and donor-specific unresponsiveness, as manifested by acceptance of a second graft from the original donor strain but rejection of third-party grafts. Similar results were obtained with donor major histocompatibility complex class I gene transfer into bone marrow cells. Gene therapy approaches to chronic rejection included gene transfer of HO-1, soluble Fas, tissue plasminogen activator, and antisense oligodeoxynucleotides specific for the anti-apoptotic mediator Bcl-x or the E2F transcription factor. Despite major experimental advances, however, gene therapy for heart transplantation has not entered the clinical arena yet. Fundamental questions regarding the most suitable vector, the best gene, and safety issues remain unanswered. Well-controlled studies that compare gene therapy with established treatments in nonhuman primates are needed before clinical trials can be started.

## *Gene Therapy for Peripheral Arterial Disease*

### Angiogenesis by Gene Therapy

Therapeutic angiogenesis is a novel strategy for the treatment of limb ischemia. Recombinant angiogenic factors such as FGF have been used to augment collateral artery development in animal models of myocardial and hind limb ischemia. Adenovirus-delivered FGF-4 (Ad5FGF-4) by intramuscular injection has been studied in a small series of patients with critical limb ischemia. Ad5FGF-4 seems safe, but transfection efficacy is limited at the assessed doses and conclusions regarding clinical efficacy cannot be drawn from the small number of patients studied (Matyas et al. 2005).

VEGF stimulates angiogenesis, and protocols for therapeutic angiogenesis by gene transfer in patients with PAD have been presented. The technique involves site-specific transfer of plasmid DNA encoding the VEGF.

### HIF-1α Gene Therapy for Peripheral Arterial Disease

Ischemia is a stimulus for production of angiogenic cytokines that activate local vascular cells and mobilize angiogenic cells to the circulation. These responses are impaired in patients with peripheral arterial disease. Hypoxia-inducible factor (HIF)-1 mediates adaptive responses to ischemia, including production of angiogenic

cytokines. HIF-1α activates a cascade of proteins associated with blood vessel formation. This comprehensive approach contrasts with angiogenic therapies employing a single protein, such as a VEGF isoform, given as a gene or as a recombinant protein.

Genzyme's engineered version of HIF-1α turns on the expression of many angiogenic proteins, including all known VEGF isoforms, angiopoietins 2 and 4, and placenta growth factor among others; it may produce a more robust and longer-lasting angiogenic effect than gene therapies based on a single growth factor. HIF-1α has been shown in preclinical studies to turn on the expression of proteins associated with the body's response to tissue ischemia, including many associated with blood vessel formation.

Genzyme had conducted phase I clinical trials of HIF-1α gene therapy in patients with critical limb ischemia (CLI). Investigators reported that Ad2/HIF-1α/VP16 appears safe, with no treatment-related serious adverse events observed in any of the patients treated, even at the highest dose. The strongest suggestion of bioactivity was complete healing of leg ulcers. In addition, all treated patients who experienced rest pain, but no ulcers at the start of the trial, had resolution of their rest pain at 1 year. The limb survival rate for treated patients was 67% versus an expected rate of 50–65% at 1 year. These improvements were observed despite significant challenges posed by the advanced nature of the patients' disease, investigators said. All patients enrolled in the trial had extensive blockage of their blood vessels prior to the trial, and many had previous bypass operations. Based on the results seen in this trial, Genzyme started a randomized double-blind placebo controlled phase II clinical trial of HIF-1α in 2005 in patients with intermittent claudication, a type of peripheral arterial disease that results in disabling pain or fatigue in the legs, brought on by exercise. In addition to safety, the trial evaluated the effectiveness of each dose in several measures of efficacy. The primary endpoint was change in the maximum amount of time a patient can walk on a treadmill without stopping due to claudication symptoms. Other endpoints include the amount of time it takes while walking for the onset of claudication pain; change in blood flow to the limbs, as measured using the ankle-brachial index; and various quality of life assessments. The primary endpoint was evaluated at 6 months and study participants were followed for 2 years. The trial is now listed as completed and inactive with no publication of the results.

### HGF Gene Therapy for Peripheral Arterial Disease

AnGes MG is developing DNA-based delivery of hepatocyte growth factor (HGF), an angiogenic factor, for indications related to PAD and IHD. It is in phase II trials in the USA and phase III trials in Japan for PAD. The DNA-based HGF therapy, combined with Vical's nonviral gene delivery technology, can be delivered with a simple injection. This is intended to cause production of HGF locally at the site of injection for an extended period. The resulting angiogenic effect is expected to alleviate ischemic disease, in which narrowed or diseased blood

vessels restrict blood flow. DNA-based HGF may be effective for people who do not respond to sufficiently conventional drugs, balloon catheterization, or surgery.

### *Maintaining Vascular Patency After Surgery*

Within the first year, 15–20% of coronary artery saphenous vein bypass grafts occlude because of thrombosis or progressive intimal hyperplasia. One strategy to reduce this complication would be the introduction of antithrombotic or antiproliferative genes in venous bypass grafts before implantation. The success of this approach requires an efficient DNA delivery system. Further studies will be needed to determine the long-term effects of this procedure on improvement of vein graft patency.

Vein graft remodeling results in reduction of the high distensibility of the vessel wall and provides capability of coping with the arterial blood pressure. This process involves formation of neointimal layer, which is very susceptible to atherosclerosis. Up to 50% of the grafts fail within a period of 10 years as a result of this occlusive disease. An intraoperative antisense approach using oligodeoxynucleotides can selectively block vascular smooth muscle proliferation and prevent accelerated atherosclerosis. HJV-liposome oligodeoxynucleotide complexes have been used for genetic engineering of vein grafts in New Zealand White rabbits. These genetically modified grafts demonstrate a sustained resistance to diet-induced atherosclerosis.

Thrombosis is a recognized complication of several surgical procedures on the arteries. Systemic anticoagulation is sometimes used to prevent this complication. One method relevant to gene therapy approach in such situations is a model for gene transfer via adventitia using a silastic collar wrapped around rabbit carotid arteries. The collar serves as a reservoir for the delivery of β-galactosidase marker gene. The following gene transfer systems were tested in this model: plasmid/liposome complexes, MMLV retroviruses, VSV (vesicular stomatitis virus)-G pseudotyped retroviruses, and adenoviruses. There was detectable gene transfer to the arterial wall including the endothelium with all delivery system. The highest transfer efficiency (10%) was seen with adenoviral vectors. The advantage of this system is that no intravascular manipulations are required. This system enables delivery of genes to the adventitia from an adventitial reservoir to prevent thrombosis during vascular procedures such as insertion of prostheses and bypass surgery.

## Antisense Therapy for Cardiovascular Disorders

Antisense molecules are synthetic segments of DNA or RNA, designed to mirror specific mRNA sequences and block protein production. The use of antisense drugs to block abnormal disease-related proteins is referred to as antisense therapeutics. Synthetic short segments of DNA or RNA are referred to as oligonucleotides. The literal meaning of this word is a polymer made of few nucleotides. Naturally occurring

**Table 8.3** Potential applications of antisense in cardiovascular disorders

| |
|---|
| Coronary restenosis following angioplasty |
| Congestive heart failure |
| Hypertension |
| Hypercholesterolemia |
| Prevention of atherosclerosis in vascular grafts |
| Prevention of arterial thrombus formation |
| Prevention of rejection following heart transplantation |



RNA or DNA oligonucleotides may or may not have antisense properties. Antisense oligonucleotides are synthetic pieces of DNA (at least 15 nucleotides in length) that can hybridize to sequences in the RNA target by Watson-Crick or Hoogstein base-pairing. Aptamers are synthetic chains of nucleotides that bind directly to target proteins, inhibiting their activity and are considered to be antisense compounds. Peptide nucleic acids (PNAs), DNA-like molecules, are potential antisense and antigene agents.

Antisense therapy is considered to be form of gene therapy because it is modulation of gene function for therapeutic purposes. However, oligonucleotides differ from standard gene therapies because they cannot give rise to proteins but can only block the expression of existing genes. Several antisense approaches use gene therapy technologies, for example, ribozymes and antisense RNA using vectors.

One approach to antisense therapy is the use of antisense oligodeoxynucleotides (ODN) that are complementary to the messenger RNA (mRNA) of interest. The second approach is the use of ribozymes, a unique class of RNA molecules that not only store information but also possess catalytic activity. Ribozymes are known to catalytically cleave specific target RNA species, leading to their degradation, whereas antisense molecules inhibit translation by binding to mRNA sequences. Thus, theoretically, ribozymes are more effective in inhibiting target-gene expression. A third approach is the transfection of cis-element double-stranded decoy ODN. Transfection of decoy ODN will result in attenuation of authentic cis-trans-interaction, leading to the removal of trans-factors from the endogenous cis-elements, with subsequent modulation of gene expression. Potential applications of antisense approach in cardiovascular diseases are listed in Table 8.3. The most important application is for coronary restenosis and this is described in Chap. 8.

## *Antisense Therapy for Hypertension*

The application of antisense oligonucleotides (AS-ODNs) for the treatment of hypertension mainly targets the renin-angiotensin system (Phillips and Kimura 2005). Other genes, such as that coding for the beta1-adrenoceptor, have also been targeted with AS-ODNs in an attempt to reduce blood pressure. Strategies for

the application of antisense technologies can be classified in two ways: the direct application of AS-ODNs and the production of AS by AS-cDNA inserted into viral vectors. Promising preclinical results from basic research have made feasible the possibility for antisense gene therapy of hypertension in the future.

## *Antisense Therapy for Hypercholesterolemia*

Antisense oligonucleotides, designed to specifically and selectively inhibit novel targets involved in cholesterol/triglycerise homeostasis, represent a new class of agents that may prove beneficial for the treatment of hyperlipidemias resulting from various genetic, metabolic, or behavioral factors.

Mipomersen (ISIS 301012), a second generation antisense oligonucleotide, selectively targets apoB-100, a large protein synthesized by the liver that plays a fundamental role in human lipoprotein metabolism cholesterol. Mipomersen lowers circulating apoB and LDL cholesterol levels (Athyros et al. 2008). Less than 1 month following dosing, healthy volunteers achieved a median reduction of 60% in apoB-100 and a median reduction of 54% in LDL. With statin-like lipid reductions via a non-statin mechanism, mipomersen's attractive profile continues to show significant promise as an alternative for patients who are not tolerating statin therapy or as add-on therapy for the lowering of cholesterol in patients failing to reach their therapeutic target. Mipomersen has been shown to decrease apoB, LDL-cholesterol, and Lpa in patients with heterozygous and homozygous familial hypercholesterolemia on maximally tolerated lipid-lowering therapy. Oral formulation of mipomersen has demonstrated oral bioavailability and pharmacological activity of the drug. ISIS has conducted a series of phase II trials to optimize dose and schedule of mipomersen as monotherapy, to explore pharmacology in combination with statins and to study the effects of mipomersen in patients with familial hypercholesterolemia. It is now in phase III development for patients with homozygous familial hypercholesterolemia by ISIS Pharmaceuticals in collaboration with Genzyme Corporation. Mipomersen shows promise as an adjunctive agent by reducing apoB-containing lipoproteins in patients at high risk of atherosclerotic cardiovascular disease who are intolerant of statins (Bell et al. 2011). Although the short-term efficacy and safety of mipomersen has been established, concern exists regarding the long-term potential for hepatic steatosis with this ASO.

ISIS-CRPRx (ISIS Pharmaceuticals) is a generation 2.2 antisense drug that targets C-reactive protein (CRP) for the treatment of cardiovascular disease and inflammation. CRP is a special type of protein produced by the liver that is only present during episodes of acute inflammation. Excessive amounts of CRP have recently been linked to coronary artery disease and it may be therapeutically beneficial to significantly decrease CRP levels in patients who are at risk for coronary event. Generation 2.2 antisense inhibitors incorporate modified chemical motifs to optimize cellular enzyme activity making them three to ten times more potent than the optimal second-generation inhibitors while they maintain the long half-life that

supports infrequent dosing. In preclinical studies, CRPRx produced dramatic suppression of liver and serum CRP levels in monkeys in which CRP was induced. A phase I clinical trial has been completed to demonstrate safety and a phase II trial is planned for testing efficacy for atrial fibrillation following cardiopulmonary bypass.

Bristol-Myers Squibb and ISIS Pharmaceuticals are collaborating to discover, develop, and commercialize novel antisense drugs targeting proprotein convertase subtilisin kexin 9 (PCSK9) for the prevention and treatment of cardiovascular disease. BMS-PCSK9Rx is an antisense drug that specifically targets proprotein convertase subtilisin/kexin type 9, or PCSK9, an important protein involved in the metabolism of cholesterol and LDL. Its role is to break down the cell surface receptor that captures LDL particles. Therefore, inhibiting PCSK9 increases the number of receptors available to remove LDL-C from the bloodstream. Genetic studies in humans have demonstrated that elevated PCSK9 can lead to severely high levels of LDL-C, whereas low PCSK9 is associated with low LDL-C levels. These observations suggest that it may be therapeutically beneficial to decrease PCSK9 levels in patients who are at risk for atherosclerosis and cardiovascular disease. BMS-PCSK9Rx could offer a new and complementary mechanism to current lipid-lowering therapies to prevent and treat cardiovascular diseases. It is in preclinical development.

### *Antisense Therapy for Preventing Occlusion of Venous Grafts in CABG*

Preclinically, the most successful of antisense therapies has been edifoligide (E2F decoy), a double-stranded oligodeoxynucleotide that binds to the transcription factor known as E2F. Recently, PRoject of Ex vivo vein GRaft Engineering via Transfection (PREVENT) III and IV demonstrated that edifoligide failed to benefit human vein grafts employed to treat lower-extremity ischemia and coronary heart disease, respectively. The clinical failure of edifoligide calls into question previous models of vein graft disease and lends credence to recent animal studies demonstrating that vein graft arterialization substantially involves the immigration into the vein graft of a variety of vascular progenitor cells. Future vein graft disease therapies will likely target not only proliferation of graft-intrinsic cells, but also immigration of graft-extrinsic cells (Cai and Freedman 2006).

## RNAi for Cardiovascular Disorders

RNA interference (RNAi) is a cellular mechanism to regulate the expression of genes and the replication of viruses. RNAi or gene silencing involves the use of a double-stranded RNA (dsRNA). Once in the cell, the dsRNAs are processed

into short, 21–23 nucleotide dsRNAs termed small interfering RNAs (siRNAs) that are used in a sequence-specific manner to recognize and destroy complementary RNAs. RNAi is described in more detail in a special report on this topic (Jain 2011a).

During the past few years, major conceptual and technical advances have been made toward the therapeutic modulation of cardiac gene expression for the treatment of cardiac diseases. These include the identification of new molecular therapy targets in cardiac disorders, often derived from genetic animal models and a better understanding of the molecular and cellular determinants of cardiac gene transfer in animal models as well as in clinical trials. The development of severe heart failure in the genetic MLP(−/−) animal model could be completely abolished by the targeted ablation of phospholamban (PL), a key regulator of cardiac calcium homeostasis. This effect of permanent germ-line PL ablation provides, in conjunction with former important work on disturbed calcium handling in the failing human heart, a rationale for the suppression of PL by antisense strategies (antisense RNAs, ribozymes, RNAi) or PL variants (Poller et al. 2004).

## *RNAi for Hypercholesterolemia*

Hepatic ABCA1 contributes to HDL plasma levels and influences lipemia following meals. An adenovirus-mediated RNAi approach has been used to test the efficiency of plasmid-based siRNA-induced knockdown of co-transfected murine ATP binding cassette transporter A1 (ABCA1). The most effective plasmid was used to generate a recombinant adenovirus (Ad) as a tool to selectively downregulate ABCA1 expression in mouse liver (Ragozin et al. 2005). In comparison to controls, Ad-anti-ABCA1 infected mice showed an approximately 50% reduction of endogenous ABCA1 and a clear upregulation of apolipoprotein E.

A chemically modified siRNAs was shown to silence an endogenous gene encoding apolipoprotein B (apoB) after intravenous injection in mice (Soutschek et al. 2004). Administration of chemically modified siRNAs resulted in silencing of the apoB mRNA in liver and jejunum, decreased plasma levels of apoB protein, and reduced total cholesterol. These siRNAs were also shown to silence human apoB in a transgenic mouse model. In the in vivo study, the mechanism of action for the siRNAs was proven to occur through RNAi-mediated mRNA degradation, and cleavage of the apoB mRNA occurred specifically at the predicted site. These findings demonstrate the therapeutic potential of siRNAs for the treatment of disease. siRNA-mediated therapeutic efficacy was demonstrated in an animal model of hypercholesterolemia. Temira Pharmaceuticals is developing ApoB SNALPs (stable nucleic acid-lipid particles), which consist of a SNALP-encapsulated siRNA designed to silence ApoB. ApoB SNALPs are delivered with high efficiency into the liver hepatocytes, the cells which produce ApoB, where the siRNA acts to knock down the precursor mRNA coding for ApoB protein. The resulting decrease in circulating VLDL and LDL results in significant reductions in LDL

cholesterol and triglycerides. In preclinical studies, rodents with diet-induced hypercholesterolemia that received a single administration of ApoB SNALP saw elevated blood cholesterol levels fall to normal. Compared to other molecular approaches in development, such as antisense, ApoB SNALP appears to have significant advantages including markedly improved drug activity. Tekmira conducted a phase I human clinical trial for TKM-ApoB in 2009 to evaluate the safety, tolerability and pharmacokinetics of escalating single doses of TKM-ApoB in patients with elevated LDL cholesterol. The trial was also designed to provide preliminary data on the ability of TKM-ApoB to lower serum LDL cholesterol levels. In 2011, Tekmira was in the process of selecting a new siRNA payload and evaluating new formulations for TKM-ApoB. Subsequent clinical studies will evaluate the safety and efficacy of ApoB SNALP as a single agent and in combination with other cholesterol-lowering drugs.

## *microRNA and the Cardiovascular System*

microRNAs (miRNAs), small and mostly non-coding RNA gene products, are molecules derived from larger segments of "precursor" RNA that are found in all diverse multicellular organisms. miRNAs are 21–25 nucleotide transcripts that repress gene function through interactions with target mRNAs The biological importance of miRNAs has been investigated in cardiovascular physiology and pathology. miRNAs expression is tightly controlled in a tissue-specific and developmental stage-specific manner and some of them are highly and specifically expressed in cardiovascular tissues. One study has shown that at least 87 miRNAs are altered in heart disease and that different types of heart disease are associated with distinct changes in miRNA expression (Ikeda et al. 2007). These data will guide further studies of the contribution of miRNAs to pathogenesis of heart disease and therapeutics based on this knowledge.

### Role of miRNAs in Angiogenesis

Embryonic lethality observed in Dicer knockout mice has been attributed to defective blood vessel formation and maintenance. These anatomical defects were associated with altered expression of VEGF, its receptors KDR (VEGFR2) and FLT-1 (VEGFR1), and the angiopoietin receptor, Tie-1 suggesting that miRNAs may regulate the expression levels of crucial angiogenic factors. Screening analysis of 168 human miRNAs revealed that members of the let-7 family including miR-21, miR-126, miR-221, and miR-222 are highly expressed in endothelial cells; thrombospondin-1, an endogenous angiogenesis inhibitor, was predicted to be a major target for the let-7 cluster (Kuehbacher et al. 2007). Deciphering the miRNA network responsible for the fine-tuning of the angiogenic process might

lead to new therapeutic approaches to modulate angiogenesis, potentially useful in ischemic conditions such as myocardial ischemia and peripheral vascular disease vascular.

### Role of miRNAs in Cardiac Hypertrophy and Failure

Diverse forms of injury and stress evoke a hypertrophic growth response in adult cardiac myocytes, which is characterized by an increase in cell size, enhanced protein synthesis, and reactivation of fetal genes, often culminating in heart failure and sudden death. Several studies have tried to establish a link between miRNAs and cardiac hypertrophy/failure. Search of miRNAs that are regulated during cardiac hypertrophy and heart failure has revealed >12 miRNAs that are up- or downregulated in cardiac tissue from mice in response to transverse aortic constriction or expression of activated calcineurin, stimuli that induce pathological cardiac remodeling (van Rooij et al. 2006). Many of these miRNAs are similarly regulated in failing human hearts. Forced overexpression of stress-inducible miRNAs was sufficient to induce hypertrophy in cultured cardiomyocytes. Similarly, cardiac overexpression of miR-195, which is upregulated during cardiac hypertrophy, results in pathological cardiac growth and heart failure in transgenic mice. miR-1 is also involved in cardiac hypertrophy and failure. Regulation of cardiomyocyte apoptosis has been suggested for miR-1 and miR-133 with miR-1 being pro-apoptotic and miR-133 being anti-apoptotic. Another miRNA, miR-21, which is upregulated in hypertrophic murine hearts, could also participate to the cardiac disease phenotype. Owing to its capacity to simultaneously regulate multiple pro-apoptotic genes and decrease apoptosis, miR21 plays a role in cardiac hypertrophy. Miragen Therapeutics is developing miRNA-based drugs for cardiac hypertrophy and heart failure.

miRNAs expression profiles, in combination to cardiac transcriptome, have been analyzed in left ventricles from fetal and failing human hearts because first, miRNAs exert regulatory functions during cardiac development and second, CHF is characterized by reactivation of a fetal gene program (Thum et al. 2007). They showed that alterations in cardiac transcriptome and miRNAs expression in heart failure both displayed a pattern strikingly similar to that observed in the fetal heart. It is likely that reexpression of fetal miRNAs during heart failure modulates a substantial fraction of the cardiac transcriptome, including the activation of a fetal gene program. These findings reveal an important role for specific miRNAs in the control of hypertrophic growth and chamber remodeling of the heart in response to pathological signaling, and point to miRNAs as potential therapeutic targets in heart disease.

### Role of miRNAs in Conduction and Rhythm Disorders of the Heart

miR-1 has a direct role in conduct in the heart muscle. miR-1-2 knockout mice, which survive to adulthood exhibit marked electrical conduction defects, including

reduced heart rate and prolonged ventricular depolarization. The cardiac transcription factor Irx5, a known regulator of cardiac repolarization, is a direct target of miR-1-2 and may be directly responsible for this electrical conduction defect (Zhao et al. 2007). Knockdown of miR-1 prevents heart arrhythmias, while miR-1 overexpression caused cardiac arrhythmias in normal and infarcted rat hearts. Both the gene encoding the cardiac gap junction channel connexin43 and the gene encoding Kir2.1, the principle pore-forming subunit for the inwardly rectifying potassium ion current IK1, are targeted by miR-1. miR-1 levels are increased in patients who have had a myocardial infarction as well as in a rat model of myocardial infarction. These results strongly suggest that a tight regulation of miR-1 levels is crucial for the maintenance of normal cardiac conduction and raise hope that miR-1 inhibition after myocardial infarction could reduce sudden death.

### miRNA-Based Approach for Reduction of Hypercholesterolemia

Hypercholesterolemia is an important risk factor for cardiovascular disease, and liver plays an important role in cholesterol metabolism. Antisense inhibition is a powerful technique in regulating the function of miRNAs in the liver. To determine the role of miR-122 in the adult liver, Isis scientists inhibited miR-122 with an antisense oligonucleotide in mice (Esau et al. 2006). The antisense inhibition of miR-122 in normal and high fat-fed mice resulted in a significant improvement in numerous metabolic and cardiovascular risk factors as evidenced by reduced plasma cholesterol levels, increased hepatic fatty acid oxidation, decreased hepatic fatty acid and cholesterol synthesis rates, and reduced fat in the liver (steatosis). These results implicate miR-122 as a key regulator of cholesterol and fatty-acid metabolism in the adult liver and suggest that miR-122 may be an attractive therapeutic target for cardiovascular and metabolic diseases.

### miRNAs as Therapeutic Targets for Cardiovascular Diseases

In the search for novel molecular entities as therapeutic targets for cardiovascular diseases, miRNAs could constitute a major breakthrough because both miRNAs and their regulatory targets are potentially druggable. Druggability of miRNAs will imply development of cell-permeable small molecules specifically regulating cardiovascular miRNAs, either mimicking or antagonizing miRNAs with hopefully subsequent normalization of gene networks. In view of the polygenic nature of a number of diseases, use of a single molecule to modulate a cluster of pathogenic genes (generally encoding proteins with related functions), appears to be an excellent idea. However, in view of the existence of several hundred miRNAs, the major challenge will be the selection of the miRNA candidates for therapeutic manipulation.

Safety issues should also be considered because some miRNAs expressed in cardiovascular tissues may also be expressed in other tissues. Moreover, a single

miRNA may target hundreds of genes and this "one hit-multiple targets" strategy might be double-edged and responsible for off-target effects. To overcome this possible drawback, suggestions have been made to make miRNA actions gene-specific, for example, miR-1/miR-133 act specifically on HCN2/HCN4 (Xiao et al. 2007). Both positive and negative cardiac actions can be attached to a same miRNA (e.g., antihypertrophic and pro-arrhythmogenic effects respectively for miR-1) because of their many downstream targets.

Finally, the identification of the predicted binding sites (in mRNAs) of disease-specific miRNAs, using computer algorithms, may point out previously unrecognized protein targets within a disease pathway of interest. On the whole, miRNAs and miRNA target predictions are likely to represent promising fields for new drug discovery.

### Future Prospects of miRNA in the Cardiovascular Therapeutics

Through the regulation of the expression of genes involved in cell growth, contractility, and electrical conductance, cardiac miRNAs may play a major role in heart development and function. In vascular cells, miRNAs have been linked to vasculoproliferative conditions such as angiogenesis and neointimal lesion formation. Diagnostic use and therapeutic modulation of individual miRNAs or miRNA clusters in cardiovascular diseases need to be further explored in the future. Molecules specifically regulating cardiovascular miRNAs, either mimicking or antagonizing miRNAs actions, will hopefully normalize dysfunctional gene networks and constitute a new therapy paradigm of cardiovascular diseases (Scalbert and Bril 2008).

Treatments focusing on miRNAs could involve delivery of antisense oligonucleotides to block existing miRNA function. The disadvantages of this approach include the difficulty in delivering enough antisense oligonucleotide or miRNA to achieve the desired therapeutic effect and potential side effects of nontargeted delivery of such pharmaceuticals. An alternative approach is to deliver gene therapy vectors expressing miRNAs to cardiomyocytes (Gray and Samulski 2008). This approach would enable external drug-mediated regulation of expression, and more than one miRNA or antagonist could be expressed from a single vector. Moreover, gene therapy vector delivery would require only one administration. The disadvantage of this approach is the use of a gene delivery vector with potential adverse effects and regulatory restrictions.

## Future Prospects of Gene Therapy of Cardiovascular Disorders

Gene therapy for cardiovascular diseases is still in its infancy but it will continue to grow. Identification of new therapeutic targets and the availability of vectors with enhanced myocardial tropism offer the opportunity for the design of gene therapies

for both protection and rescue of the myocardium. Availability of genomic screening technologies will help in the identification of novel therapeutic targets and detection of disease-causing polymorphisms, which may lead to the design of individualized gene and cell-based therapies (Melo et al. 2004). Particular attention should be paid to the following aspects:

- Gene therapy, though feasible for the treatment of dyslipoproteinimia and atherosclerosis, would have a limited impact on multifactorial diseases.
- Gene transfer methods need to be refined and adopted for use for various targets in the cardiovascular system.

By the year 2015, new technology using stable gene integration may lead to the development of more effective and lifelong therapy for diabetes, familial homozygous hypercholesterolemia, and other acquired diseases. It is unlikely that these gene therapy applications will be used routinely in clinical practice within the next few years because of the time taken for the regulatory process to be completed and for physicians to adopt the technologies in their daily practice. However, by 2015, some of the studies will have resulted in products that would enter regular clinical use. It is possible, for example, that FGF-4 will be used in conditions such as chronic stable angina refractory to medical therapy and in patients with evidence of coronary occlusion. New trials of gene- and cell-based therapy will be in progress, and many that will provide important treatment directions for the future will be completed. Between 2010 and 2015, there is expected to be an emphasis on aggressive reduction in risk factors, which will be achieved by the introduction of new drugs. The trend will be toward earlier coronary interventional therapy. New surgical techniques may also have emerged. Within this time frame, it is conceivable that angiogenic gene therapy will be used in selected populations. Small molecules, DNA-decoy techniques, or gene-based therapy will be used to inhibit the cell cycle in order to prolong the functional life of bypass grafts. Autologous endothelial cell harvesting may even be used to produce an engineered graft. Further research will provide a rationale for using cell-based therapy to repair vascular and myocardial tissue. Long-term myocardial protection from ischemia and/or reperfusion using preemptive gene therapy will be in the advanced phase of investigation. Parallel developments in the field of genetic biomarkers, genomics, and proteomics will help to pinpoint drug targets and identify patient populations to be treated by these technologies within the framework of personalized medicine.

## Companies Involved in Gene Therapy of Cardiovascular Disorders

Companies involved in gene therapy of cardiovascular disorders are shown in Table 8.4.

**Table 8.4** Companies involved in gene therapy of cardiovascular diseases

| Company | Technology/product/applications | Stage |
|---|---|---|
| AnGes MG Inc | Hepatocyte growth factor for angiogenesis/ PVD and CAD | Filed in Japan |
| Ark Therapeutics | Trinam™: consists of a local delivery device and a gene-based medicine to prevent thrombosis of veins and arteries after | Phase II/III |
| Berlex (now part of Bayer Healthcare) | Ad5FGF-4 angiogenic gene therapy product for the treatment of patients with stable exertional angina due to CAD | Phase IIB/III halted |
| BioCardia Inc | Catheter for percutaneous intramyocardial gene delivery | Research |
| Biosense Webster/ Sanofi-Aventis | The NOGA system is catheter-based system for intramyocardial navigation to deliver angiogenesis gene therapy (FGF1) to treat cardiovascular disease | Phase II |
| Cardium | GENERX (Ad5-FGF4) for gene therapy of patients with angina due to CAD | Phase II/III on going |
| Celladon Corporation/ Targeted Genetics Corp | MYDICAR® targeting SERCA2a: AAV-based gene therapy for CHF | Phase I/II |
| Cordis Corporation (Johnson & Johnson) | NOGA® Cardiac Navigation System for delivery of cardiac cell and gene therapies | Marketed |
| Expression Genetics | Water-soluble lipopolymer is a local or systemic gene therapy delivery system to prevent and treat cardiovascular diseases | Preclinical |
| Genzyme | GeneGraft, based on engineered HIF-1α gene, to promote angiogenesis in PAD and IHD | Phase I |
| | The proprietary gene therapy BARKct prevents congestive heart failure and reverses prior damage to heart tissue | Preclinical |
| NanoCor Therapeutics | Targeted noninvasive delivery of a gene, Carfostin®, with BNP™ (Biological NanoParticle) and rAAV for treatment of CHF | Preclinical |
| Sanofi-Aventis | Temusi® (NV1FGF; riferminogen pecaplasmid): nonviral plasmid-based local IM gene-delivery for FGF-1 angiogenic therapy for critical limb ischemia | Phase III failed |
| ViroMed Co Ltd/ Takara Bio | Gene-based drug, VMDA3601 for patients suffering from critical limb ischemia and IHD | Phase I |

*Abbreviations*: *CAD* coronary artery disease, *PAD* peripheral arterial disease, *IHD* ischemic heart disease, *CHF* congestive heart failure

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# Chapter 9
# Coronary Angioplasty and Drug-Eluting Stents

## Introduction

Drug-eluting stents (DES) are one of the most important devices in cardiovascular drug delivery. Since the introduction of these stents in 2002, more than 2 million have been implanted worldwide. DES are metal stents that are coated with a polymer containing an antiproliferative agent, which is released gradually over the course of weeks to months after the stent is inserted, thereby providing sustained inhibition of the neointimal proliferation as a response to vascular injury, which is responsible for restenosis. This chapter will start with an evolution of DES from angioplasty and stents.

### *Percutaneous Transluminal Coronary Angioplasty*

Percutaneous transluminal coronary angioplasty (PTCA) is used to widen the stenosis of the coronary without an open surgical procedure. It is also applied for the treatment of stenosis of the carotid arteries that supply blood to the brain. It is carried out by positioning a catheter with a small inflatable balloon on the end within the narrowed portion of the artery. The balloon is then inflated, which dilates the stenosed segment of the artery by compressing the atherosclerotic plaque and stretching the wall of the artery. This procedure is usually followed by insertion of a stent at the site of stenosis.

Approximately 1.5 million patients undergo percutaneous coronary intervention in the USA every year. Depending on local practices and the diagnostic criteria used, 5–30% of these patients (75,000–450,000) have evidence of a periprocedural myocardial infarction (MI). According to a universal definition of MI, cardiac biomarker (preferably cardiac troponin) level, which is more than three times the upper reference limit is indicative of a periprocedural MI (Thygesen et al. 2007). At the higher estimate, the incidence of these events is similar to the annual rate of major spontaneous MI. Mechanism of this complication and methods for prevention as well as management have been reviewed elsewhere (Prasad and Herrmann 2011).

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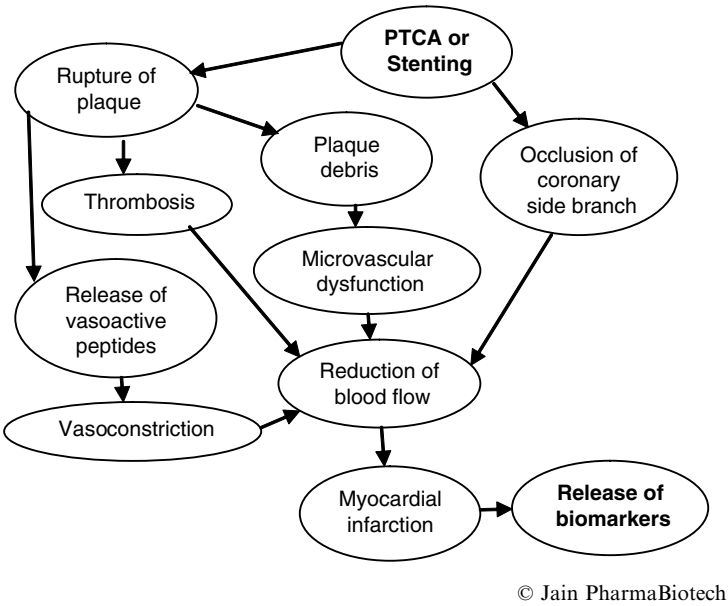


**Fig. 9.1** Myocardial infarction following procedures on coronary arteries

Release of biomarkers of myocardial injury following periprocedural MI is schematically depicted in Fig. 9.1.

### *Stents*

A coronary stent is a tiny expandable mesh tube made of medical grade stainless steel. A stent is delivered on a balloon catheter and implanted in the coronary artery after balloon angioplasty to help keep the artery open. After the plaque is compressed against the arterial wall, the stent is fully expanded into position, thereby acting as miniature "scaffolding" for the artery. The balloon is then deflated and removed and the coronary stent is left behind in the patient's blood vessel. It may be necessary to place more than one stent, depending on the length of the blockage. The inside lining of the artery eventually heals around the stent. Restenosis after stenting and its treatment is described later in this chapter.

## Restenosis

Restenosis – the reclosure or narrowing of an artery – can be a concern with coronary stents and with balloon angioplasty procedures. Restenosis occurs when blockages return a few weeks or months after the coronary stent procedure.

Abnormal growth of smooth muscle lining the arterial walls plays an important role in vascular occlusion during CAD. It also contributes to the blockage of coronary arteries that have been opened by balloon angioplasty or replaced in bypass operations. Although the initial success rate of PTCA for opening obstructed coronary arteries reaches 95%, restenosis occurs at the site of angioplasty. Of the estimated 1 million stents implanted annually in major markets, approximately 20–25% of patients treated with conventional, bare metal stents need a second procedure within 6 months because the vessel can develop reblockage.

This is a big clinical and economic problem because the number of PTCAs is increasing in recent years. Long-term failure negate the beneficial effects of PTCA and after three unsuccessful PTCAs, the patient needs a coronary artery bypass operation. Development in coronary stenting is changing the picture. Apart from improved mechanical design, the stents are being coated with anything from heparin and antiplatelet drugs to growth factor inhibiting agents to prevent restenosis. The two potential complications, thrombosis and intimal hyperplasia, still remain issues that need to be addressed.

## *Pathomechanism*

The mechanism of restenosis following angioplasty has been studied intensively. The primary pathophysiological mechanism involves an exaggerated healing response of the smooth muscle to vascular injury. Injury induces smooth muscle cells to proliferate and migrate to the subintimal layer, where the smooth muscle continues to proliferate and secrete extracellular matrix. These processes cause the neointimal mass to expand and gradually encroach on the coronary lumen. Various explanations of this are:

1. Atherosclerosis is sometimes considered to be a form of benign neoplasia.
2. Smooth muscle cells derived from restenotic lesions show a higher intrinsic migratory activity than cells derived from primary lesions. A lesion that has a higher proportion of these biologically aggressive cells will develop more stenosis than a lesion without similar biological characteristics.
3. Removal of inhibitory influences. Endothelial cells normally secrete nitric oxide (NO) and heparan sulfate both of which inhibit smooth muscle cell proliferation. Their removal by mechanical trauma of angioplasty contributes to a proliferative environment.
4. Induction of stimulatory influences. Platelets adhere to the injured vessel and cause a thrombus to develop at the site of injury. Free radicals and various cytokines released during the vascular injury increase the expression of cell adhesion molecules.
5. Activation of cell signal pathways induces the cell to move from a quiescent to a proliferative state. Enzyme cyclin-dependent kinase 2 (cdk 2) may play an important role in neointima formation.

## *Treatment*

Treatment for limiting restenosis is shown in Table 9.1.

## *Role of NO in the Management of Coronary Restenosis*

One of the major problems related to the percutaneous transluminal coronary angioplasty technique is the renarrowing of the vessel, a phenomenon known as restenosis. NO and nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to play a role in this pathology. NO counteracts several mechanisms involved in the restenotic process after coronary angioplasty. During restenosis, an impairment of NO-dependent pathways may occur. NO mediates an antiproliferative effect on smooth muscle cells, inhibition of leukocyte–vessel wall interactions, and platelet aggregation and adhesion. Because these effects are mainly dose dependent, NO-releasing drugs have to be applied at a high dose to have an effect on restenotic mechanisms. The main problem with the use of conventional NO donors is that they affect blood pressure and flow, and for these reasons, they cannot be used safely in clinical practice. Various novel methods for NO delivery have been investigated including use of modified NO donors, NO-releasing stents, and eNOS gene therapy.

### Modified NO Donors

Treatment with the NO donor molsidomine at a high dose for 6 months after coronary angioplasty has no effect on the angiographic restenosis rate. Due to the vasodilating effect of NO, the anginal status improves slightly more in patients receiving molsidomine.

**Table 9.1** Treatment of restenosis

| |
|---|
| *Pharmacological agents* |
| ACE inhibitors |
| Anticoagulants and thrombolytics |
| Antioxidants: probucol |
| Antiplatelet agents |
| Calcium channel blockers |
| Cytotoxic drugs |
| Growth factor inhibitors |
| Inflammatory mediator inhibitors |
| Lipid lowering agents |
| Monoclonal antibody: abciximab |
| Nitric oxide (NO)-based therapies and combination of NO with carbon monoxide |
| *Radiation* |
| *Antisense therapy* |
| *Gene therapy* |
| *Stents and other devices* |

NCX 6560 (NicOx), a modified NO-donor, is in clinical development for cardiovascular diseases. In the first study on both healthy male volunteers and those with abnormally raised cholesterol, NCX 6560 will be compared to placebo and atorvastatin (Lipitor®), with a preliminary evaluation of activity, safety, and tolerability. Promising preclinical results suggest that NCX 6560 could inhibit multiple steps in the development of atherosclerosis, a key dysfunction underlying cardiovascular disorders.

## NO-Generating Stents for Coronary Restenosis

Reduced or impaired synthesis of NO promotes the proliferation of vascular smooth muscle cells, and may thus induce the neointimal formation leading to coronary in-stent restenosis. Glu298Asp polymorphism in exon 7 of the endothelial NOS gene is associated with coronary spasm and acute myocardial infarction.

During angioplasty and stenting, there is damage to the delicate endothelial layer that normally generates NO and prevents smooth muscle cell proliferation. This starts a vicious circle shown in Fig. 9.2. Thrombus formation and eventual intimal hyperplasia are the leading causes of small-diameter synthetic vascular graft failure.

To counteract this, restenosis intrapericardial delivery of NO donors for which NO release rates and pericardial residence times are matched and optimized might be a beneficial adjunct to coronary angioplasty. A gold-plated stent may be used, which should bring about the release of endogenous NO.

$$\mathrm{Au} + \mathrm{RSNO} \rightarrow \mathrm{Au^{I}} - \mathrm{SR} + \mathrm{NO}$$

Incorporation of a diazeniumdiolate-modified NO-producing peptide into polyurethane improves the thromboresistance of a biocompatible polymer vascular graft (Jun et al. 2005). NO production by polyurethane films continues for approximately 2 months under physiological conditions, and mechanical properties of the material are suitable for vascular graft applications. Platelet adhesion to NO-releasing polyurethane is dramatically decreased compared to control

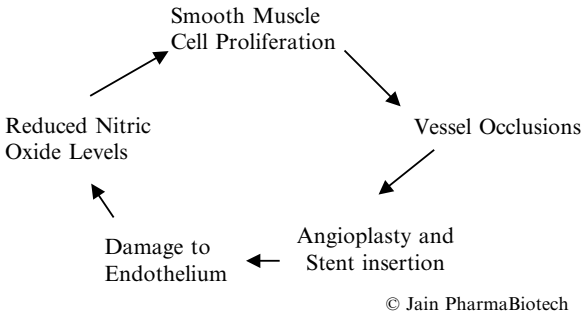


**Fig. 9.2** Vicious circle of vascular occlusion following angioplasty and stenting

polyurethane. Furthermore, endothelial cell growth is stimulated in the presence of the NO-releasing polyurethane, while smooth muscle cell growth is greatly inhibited. The ability of this bioactive material to inhibit platelet adhesion and smooth muscle cell proliferation while encouraging endothelialization suggests that this NO-generating polyurethane may be suitable as a candidate material for small-diameter vascular grafts. Early reendothelialization and inhibition of platelet aggregation should enhance long-term patency by establishing an endothelial environment resembling that of normal healthy vessels. The endothelial cells that overlie atherosclerotic plaques in diseased blood vessels do not produce much biologically active NO because they express little eNOS. The therapeutic potential of NO-eluting polyurethane grafts may therefore lie in the hope that they more closely mirror a healthy vascular endothelial phenotype (Verma and Marsden 2005).

The closely related topic of drug-eluting stents is described in detail in a special report on cardiovascular drug delivery (Jain 2011b). Nanotechnology has also been incorporated into drug-eluting stents and is discussed in a report on nanobiotechnology (Jain 2011a).

## *Carbon Monoxide Inhalation for Preventing Restenosis*

Carbon monoxide (CO), a chemically stable gas, occurs in nature as a product of the oxidation or combustion of organic materials. CO also arises in cells and tissues as a byproduct of heme oxygenase (HO), a rate-limiting enzyme that degrades heme into CO, iron, and bilirubin. CO acts as a vasorelaxant and may regulate other vascular functions such as platelet aggregation and smooth muscle proliferation. Many of the effects of CO depend on the activation of guanylate cyclase, which generates guanosine 3′,5′-monophosphate (cGMP), and the modulation of mitogen-activated protein kinase (MAPK) signaling pathways. Actions of CO on the human body range from the physiological to the pathological: small amounts may be beneficial but large amounts are toxic.

Veins are considered to be the best conduits available for CABG surgery. When these arterialized vein grafts fail, it is often due to the development of intimal hyperplasia. Ex vivo treatment of vein grafts in CO-saturated lactated Ringer solution has been shown to preserve vascular endothelial cell integrity perioperatively and significantly reduces neointima formation (Nakao et al. 2010). These effects appear to be mediated through the activation of the HIF1α/VEGF pathway. CO is an effective means of reducing intimal hyperplasia in large animals after vascular injury when delivered during the operative procedure (Raman et al. 2006). No toxicity is associated with the increase in COHgb. The combination of CO and NO provides additional protection against the vascular injury response, with a greater reduction in neointima formation. These data suggest that these agents may prove to be clinically beneficial in prolonging vascular patency after interventions.

## *Antisense Approaches for Prevention of Restenosis After Angioplasty*

The following antisense experimental approaches have been shown to inhibit restenosis:

- Antisense oligonucleotide directed against cdk 2 kinase. A single application of this compound by intraluminal application can prevent neointima formation after balloon injury in rat carotid artery model.
- Antisense c-myc applied as a polymer matrix inhibits restenosis. This is based on the observation that human SMC proliferation is associated with an increase in c-myc mRNA expression.
- Antisense c-myb applied as a polymer matrix inhibits restenosis.
- Antisense PCNA (proliferating cell nuclear antigen) administered with HVJ-liposome inhibits restenosis.
- Use of antisense ODNs complexed with HVJ-liposome to E2F binding sites for inhibiting smooth muscle proliferation.

Phosphorylcholine (PC)-coated stents can be used to load and release small decoy oligonucleotides (ODNs). Despite successful ex vivo ODN deposition and nuclear uptake in the vessel wall, in vivo vascular ODN transfer has not been achieved. Rapid intravascular release of ODN before implantation and potential vascular barriers for gene transfer are most likely responsible for the currently unsatisfactory in vivo release kinetics (Radke et al. 2005).

The first clinical study demonstrated the safety and feasibility of local delivery of antisense in the treatment and prevention of restenosis; another randomized clinical trial (AVAIL) with local delivery of c-myc morpholino compound in patients with coronary artery disease demonstrated its long-term effect on reducing neointimal formation, as well as its safety. These preliminary findings from the small cohort of patients require confirmation in a larger trial utilizing more sophisticated drug-eluting technologies (Kipshidze et al. 2005). Further identification of new transcriptional factors and signaling mediators would be an important step in the development of new potential targets for therapy of vascular restenosis.

## *miRNA-Based Approach for Restenosis Following Angioplasty*

miR143/145 gene cluster promotes acquisition of the contractile phenotype of murine vascular smooth muscle cells (VSMCs). These VSMC-restricted miRNAs, which target unique combinations of SMC genes, provide an efficient mechanism to fine-tune cardiovascular homeostasis and the response of the vessel wall to injury. VSMCs from miR-143/145-deficient mice are locked in the synthetic state, which incapacitates their contractile abilities and favors neointimal lesion development. miR-143/145 targets include angiotensin-converting enzyme (ACE),

which might affect both the synthetic phenotype and contractile functions of VSMCs. Pharmacological inhibition of either ACE or the AT1 receptor partially reverses vascular dysfunction and normalized gene expression in miR-143/145-deficient mice. Manipulation of miR-143/145 expression may offer a new approach for influencing vascular repair and attenuating arteriosclerosis (Boettger et al. 2009). This important discovery will open the door to new avenues of investigation and potentially future therapies for restenosis following angioplasty.

## *Gene Therapy to Prevent Restenosis After Angioplasty*

Some of the targets for gene-based therapies of restenosis are:

- Prevention of thrombosis by inhibition of platelet effects and fibrin deposit.
- Inhibition of cytokines which downregulate proliferation. This requires identification of appropriate receptor antagonists.
- Cytostatic approaches to block cell cycling.
- Arterial regeneration by endothelial proliferation. The aim is to restore endothelium, and intimal hyperplasia should be avoided.
- Antiatherosclerotic gene transfer to reverse disease progression.
- Adenovirus-encoded ribozyme to PDGF to inhibit neointima formation after arterial injury.

Inhibition of various factors producing restenosis can be achieved by delivering genes expressing dominant negative gene products. Included among such genes are NO synthase, tPA, thymidine kinase, PGHS, and p21.

Prostacyclin (PGI2) is one of the most potent inhibitors of platelet activation, secretion, and aggregation, as well as a potent vasodilator. Its biosynthesis in vessel walls is catalyzed by phospholipase A2 (PLA2) prostaglandin H synthase (PGHS). PGHS is severely inactivated during catalysis. At sites of vascular injury, its quantity is further reduced and the capacity of PGI2 synthesis is compromised. Adenoviral vectors have been used for direct delivery of PGHS-1 genes to enhance the synthesis of vasoprotective molecules. The effectiveness of PGHS-1 transfer in reducing the thrombosis and intimal hyperplasia was demonstrated by infusion of Ad-PGHS through perforated catheters into balloon-injured pig carotid arteries.

Ras protein is a transducer of mitogenic signals. Inhibition of ras should be useful for therapy of vascular proliferative disorders. Ras inhibition by dominant negative mutants led to significant reduction of neointima formation after experimental blood vessel injury in a rat model.

Retinoblastoma gene product (Rb) is present in arterial smooth muscle cells (SMC) and inhibits cell proliferation. However, it is rapidly inactivated by phosphorylation. Adenovirus-mediated transfer of constitutionally active form of Rb at

the time of angioplasty in animal models has been shown to be sufficient to inhibit vascular SMC proliferation and neointimal formation significantly.

P21 and possibly related cyclin-dependent kinase inhibitors may normally regulate cellular proliferation following arterial injury, and strategies to increase its expression may prove therapeutically beneficial in vascular diseases. P21 is a downstream target of the p53 tumor suppressor gene and has been implicated in abnormalities of cell proliferation. Expression of p21 directly inhibits the kinase activities of cyclin/CDK complexes in vitro. P21 also inhibits proliferating cell nuclear antigen (PCNA)-dependent DNA replication but not DNA repair in vitro. Localized arterial infection with a p21-encoding adenovirus at the time of balloon angioplasty significantly reduces neonatal intimal hyperplasia in the rat carotid artery model of restenosis. Direct gene transfer of p21 using adenoviral vector into balloon-injured porcine arteries inhibits development of intimal hyperplasia.

Another potential strategy is adenoviral-mediated delivery of gene encoding herpes simplex thymidine kinase (HSV-tk). When HSV-tk is expressed in human cells, cell proliferation can be inhibited by exposure of the cells to ganciclovir, a nucleoside analogue. HSV-tk phosphorylates ganciclovir forms a nucleotide analog, which is incorporated into the elongating DNA strand, and inhibits further DNA synthesis. Adenoviral-mediated transfer and expression of HSV-tk in balloon-injured femoral artery of hyperlipidemic rabbit results in reduction in intimal proliferation. Inhibition of cell proliferation in atherosclerotic arteries constitutes a possible treatment for vascular proliferative disease in human patients.

Interferon gamma (IFN-$\gamma$), a product of T lymphocytes, found in atherosclerotic lesions, inhibits smooth muscle cell proliferation in vitro. Transfer and expression of the IFN-$\gamma$ gene in human endothelial cells has been shown to inhibit vascular smooth muscle cell growth in vitro. This may be a useful therapy for restenosis after angioplasty.

The gax (growth arrest-specific homeobox) is a potentially important gene in the process of smooth muscle cell differentiation. That gax is important for the development of restenosis has been demonstrated in animal models of balloon injury where expression of gax is rapidly downregulated as cell proliferation increases. Investigations are in progress to determine the effect of increasing expression of gax in animal models by gene therapy. This may have potential applications in management of restenosis.

Pharmacokinetics of adenoviral-mediated gene delivery to the vascular smooth muscle cells can be modulated by agents such as polaxamer 407 (a viscous biocompatible polyol), which may improve gene delivery by maintaining pericellular concentrations of vector. This technique may allow desired levels of gene transfer with lower total viral dose and exposure time. Because of the multiplicity of the factors involved in restenosis, a cocktail of genes that affect different aspects of restenosis may have to be used. The adenovirus-encoded ribozyme to PDGF A-chain mRNA has been shown to inhibit neointimal formation in a rat carotid artery injury model and this may serve as a novel strategy to prevent restenosis after coronary angioplasty.

### Techniques of Gene Therapy for Restenosis

Recombinant adenoviral vectors continue to be the most efficient methods of gene transfer into the arterial wall but concerns over the safety of using viral vectors in a clinical situation have inspired the considerable progress that has been made in improving both viral and nonviral modes of gene transfer. Early clinical trials have shown that plasmid- and adenovirus-mediated vascular gene transfers can be conducted safely and are well tolerated. Ex vivo gene therapy with E2F-decoy has succeeded in reducing graft occlusion rate after surgical bypass in a randomized, double-blind clinical trial.

An intravascular approach using a catheter is the most commonly used gene delivery method. Various types of catheters, such as microporous gel-coated and channel balloon catheters are available for gene transfer into vessel wall. Adenoviruses can be prevented from being swept away by the blood stream by double-balloon catheters but the circulation through the arteries may have to be interrupted for longer than is considered to be safe.

The efficacy of gene transfer through atherosclerotic lesions is low. Therefore, needle catheters have been devised that perform the injection from inside of the vessel lumen through the atherosclerotic lesion so that the genes are delivered directly to the vessel wall. Gene transfer vectors can also be delivered to the artery adventitia with a biodegradable collar, biodegradable gel, or a direct injection into the adventitia.

### NOS Gene Therapy for Restenosis

Endogenous NO in the vasculature is vasoprotective by inhibiting platelet and leukocyte adhesion, inhibiting SMC proliferation and migration, and promoting endothelial survival and proliferation. At sites of vascular injury following angioplasty, the endothelium is disrupted and NO synthesis is impaired. Hence, augmenting local NO synthesis through NOS gene transfer may help arrest the proliferative response to vascular injury. Delivery of eNOS gene to balloon-injured rat carotid arteries using HVJ-liposomes results in 70% reduction in neointimal formation 2 weeks after balloon injury. SMCs engineered to express eNOS using retrovirus onto the luminal surface of balloon-injured rat carotid arteries also inhibit neointimal formation.

Other studies have similarly shown that adenoviral delivery of eNOS to balloon-injured rat and porcine arteries can limit intimal hyperplasia. The effect of eNOS and plasminogen activator inhibitor 1 (PAI-1) gene transfer on neointimal formation was compared in balloon-injured porcine coronary arteries. Adenoviral-mediated eNOS, but not PAI-1 gene transfer, could significantly inhibit the neointimal formation at 4 weeks after intramural gene delivery. No acute systemic toxicity was observed after gene transfer. The results suggest that local gene transfer of eNOS may hold promise as a safe and effective adjunctive treatment to prevent postangioplasty restenosis. In addition to constitutive eNOS gene transfer, iNOS gene transfer has been investigated in balloon-injured arteries. Adenoviral-mediated iNOS gene transfer to

injured rat carotid artery using 100- to 1,000-fold lower virus concentrations than most other vascular gene therapy studies resulted in a near complete reduction (95%) in neointimal formation up to 6 weeks post injury. However, iNOS gene transfer does not result in regression of preformed neointimal lesions when gene transfer is carried out several days after balloon injury, suggesting gene therapy during the early stage of vessel injury is a determining factor for a successful outcome. Transfer of iNOS gene in this setting may be advantageous over eNOS or nNOS transduction since a substantially lower amount of adenovirus encoding iNOS seems to produce a therapeutic effect. Endothelial progenitor cells, transduced with retroviral vectors expressing human eNOS, were transplanted in the lining of rabbit carotid artery following balloon angioplasty (Kong et al. 2004). Inhibition of intimal hyperplasia reduced restenosis that follows angioplasty.

#### Nonviral Gene Therapy to Prevent Intimal Hyperplasia

A modular, self-assembling, nonviral system has been developed consisting of lipofectin, integrin-targeting peptides, and plasmid DNA (LID) and applied to a rat carotid angioplasty model of vascular injury (Meng et al. 2006). Marker gene studies identified transfection of adventitial cells after vector delivery to that layer. Human tissue inhibitor of metalloproteinase-1 (hTIMP-1) was tested as a therapeutic gene product after direct application to the exposed adventitial layer. Vascular LID.hTIMP-1 transfection was confirmed by PCR and gene expression by immunohistochemistry. There was considerable reduction in neointimal hyperplasia that followed balloon dilatation. Neointima-to-media ratios were similarly reduced. This is an effective system of therapeutic gene transfer, particularly targeting the arterial adventitia, where transfer of genes involved in matrix remodeling and cell migration may be useful.

#### HSV-1 Gene Therapy to Prevent Intimal Hyperplasia

A brief exposure of the arterial lumen to a genetically engineered, attenuated herpes simplex virus 1 (HSV-1) blocks activation of caspase 3-dependent apoptosis and MAPK-dependent cell proliferation induced by carotid artery balloon angioplasty and ligation to reduce blood flow (Skelly et al. 2007). The procedure enables the restoration of the endothelial cell layer lining the lumen and prevents neointimal hyperplasia and restenosis. These findings have a broad application in prevention of balloon angioplasty-induced restenosis.

### *Drug Delivery Devices for Restenosis*

Devices used for drug delivery in restenosis following angioplasty are shown in Table 9.2.

**Table 9.2** Devices used for drug delivery in restenosis

| |
|---|
| *Catheter-based drug delivery* |
| *Perivascular implants* |
| Biodegradable implants containing heparin |
| Intraluminal delivery of antiproliferative drugs encapsulated in biodegradable nanoparticles |
| *Intravascular stents* |
| Biodegradable polymers |
| Absorbable metal stents |
| *Drug-eluting stents (DES)* |
| Drug-coated stents |
| DES with polymer surfaces used for controlled drug release |
| Nanotechnology-based DES |
| Absorbable DES |
| *Polymeric endoneural gel paving* |
| Catheter-based drug delivery |
| Cell targeting strategies |
| Controlled release matrices |
| Facilitated diffusion: iontopheric balloon |
| Fibrin adhesive |
| Liposomes |
| Nanoparticles |
| Platelet blockage |
| Receptor-mediated cytotoxicity |
| Receptor-mediated gene transfer |
| *Gene therapy for restenosis* |
| Catheter-based gene delivery |
| Stent-based gene delivery |

## *Local Drug Delivery by Catheter*

The rationale for local drug delivery in restenosis is that local concentrations can be achieved without systemic toxicity. Catheter-based drug delivery is described in Chap. 2 and can be used for restenosis, for example, by a double balloon catheter where proximal and distal inflation isolate a vascular segment allowing instillation of drugs. These can be used for gene vectors and cell targeting strategies such as receptor-mediated gene transfer. A number of percutaneous catheter systems have been used in animal models with limited success. Recent results in human beings suggest that transfer of regulatory genes may provide a novel therapy option by inhibiting restenosis.

## *Absorbable Metal Stents*

Preliminary 3-month follow-up of a recently developed absorbable metal stent (AMS) for treatment of PAD patients with critical limb ischemia suggests a potentially promising performance of these devices in the treatment of below-knee lesions (Peeters et al. 2005). One point to consider is the possible success of these devices in preventing restenosis, which would obviate the need for DES.

## Drug-Eluting Stents

DES is the term is applied to coated stents which elute drugs to prevent restenosis. Bare stents now are being coated with a number of different agents in an attempt to inhibit restenosis. Drugs that have been investigated include the following:

- ABT-578 (zotarolimus)
- Angiopeptin
- AP23573 (mTOR inhibitor)
- Cytochalasin A
- Dexamethasone
- Estradiol
- Everolimus
- Heparin
- Latrunculin D
- Nanotechnology-based DES
- Nitric oxide
- Paclitaxel
- Prostaglandin E
- Radioactive compounds, for example, $P^{32}$ beta-emitter
- Sirolimus
- Tacrolimus (FK506)

### *Various Types of DES*

#### CYPHER® Sirolimus-Eluting Coronary Stent

The CYPHER® sirolimus-eluting coronary stent (Cordis) provides a scaffold to open the artery and slowly elutes sirolimus, an antirejection medication, that helps limit the overgrowth of normal cells while the artery heals. The procedure to place a CYPHER® stent is the same as for an uncoated stent. In a single-blind, multicenter, prospectively randomized trial on selected patients with acute myocardial infarction, the use of sirolimus-eluting stents significantly reduced the rate of target-vessel revascularization at 1 year (Spaulding et al. 2006).

In 2006, the FDA approved revised instructions for use for the CYPHER® Sirolimus-eluting Coronary Stent. The label retains its current precaution statements for multiple CYPHER® Stents but now reflects FDA's review of clinical trial data that suggests there is no increased risk of myocardial infarction (heart attack) with the use of overlapping CYPHER® Stents in comparison to bare metal stents. Overlapping stents are most often used in patients with complex coronary artery disease in which the blockage is too long for a single stent. Today, approximately 25% of stenting procedures involve the use of overlapping coronary stents. The CYPHER® Stent is the only drug-eluting stent with this new label. The revised labeling can be found at: www.cypherusa.com. This labeling change is based on a retrospective analysis of several prospective randomized controlled clinical studies

of overlapping CYPHER® Stents examining more than 900 patients. The trials and registries include SIRIUS (SIRolImUS-coated BX VELOCITY® Balloon Expandable Stent in the treatment of patients with de novo coronary artery lesions), E-SIRIUS (European version of SIRIUS), C-SIRIUS (Canadian version of SIRIUS), DIRECT (DIRECT stenting using the Sirolimus-eluting BX VELOCITY® Stent), and SVELTE (Study in Patients with De Novo Coronary Artery Lesions in Small Vessels Treated with the CYPHER® Stent). CYPHER SELECT™ Sirolimus-eluting Stent has the CE Mark in Europe for use in the treatment of severe claudication and critical limb ischemia of infrapopliteal lesions – the most severe form of arterial disease in the leg.

### Sirolimus-Eluting Versus Paclitaxel-Eluting Stents

Studies have suggested that the most promising of the drugs used in DES at the present time are sirolimus (Rapamycin®, Wyeth-Ayerst), a naturally occurring macrocyclic antibiotic, which is used as a potent immunosuppressive agent to prevent organ transplant rejection, and paclitaxel (Taxol®, Bristol-Myers Squibb), a well-known antimetabolic agent used in treatment of a number of cancers. Compared with bare metal stents, sirolimus-eluting and paclitaxel-eluting stents have been shown to markedly improve angiographic and clinical outcomes after percutaneous coronary revascularization. The finding that the TAXUS Moderate Release stent system for delivery of paclitaxel is safe and effective in the treatment of long, complex coronary artery lesions provides the evidence base for the more widespread use of DES in contemporary clinical practice (Dawkins et al. 2005).

A randomized, controlled, single-blind trial in patients undergoing percutaneous coronary intervention showed that the use of sirolimus-eluting stents results in fewer major adverse cardiac events, primarily by decreasing the rates of clinical and angiographic restenosis (Windecker et al. 2005). In a randomized trial on patients with diabetes mellitus and coronary artery disease, use of the sirolimus-eluting stent was associated with a decrease in the extent of late luminal loss, as compared with use of the paclitaxel-eluting stent, suggesting a reduced risk of restenosis (Dibra et al. 2005). Another study has compared the immediate and mid-term outcomes of sirolimus-eluting stents and paclitaxel-eluting stents in lesions of the unprotected left main coronary artery (Lee et al. 2005). Both groups exhibited excellent in-hospital and 6-month outcomes with no significant differences between them.

In a randomized trial of DES in small coronary arteries (diameter <2.8 mm), angiographic restenosis was found in 19.0% of the lesions in the paclitaxel-eluting stent group and 11.4% of the lesions in the sirolimus-eluting stent group (Mehilli et al. 2006). The results show that paclitaxel-eluting stent is associated with a greater late luminal loss and is less effective in reducing restenosis in small coronary vessels than the sirolimus-eluting stent. However, in a prospective randomized multicenter trial comparing sirolimus- and paclitaxel-eluting coronary stents, there were no differences in the rates of binary restenosis or major adverse cardiac events (Morice et al. 2006).

### Paclitaxel-Eluting Stents

In a randomized trial, the use of paclitaxel-eluting stents in acute myocardial infarction with ST-segment elevation reduced the incidence of serious adverse cardiac events at 1 year by 4% points, as compared with uncoated stents, but the difference was not statistically significant (Laarman et al. 2006).

The recanalization of a chronic total coronary occlusion (CTO) is hampered by a high rate of lesion recurrence. One study has assessed the effect of paclitaxel-eluting stents in CTOs in a strategy of extensive stent coverage and the optional use of additional bare metal stents (Werner et al. 2006). CTO was successfully recanalized with implantation of one or more Taxus stents and verified by repeat angiography. The results showed 80% reduction of target vessel failure as compared to bare metal stents, with a lower risk of late reocclusions without increased acute adverse events, demonstrating the benefit of paclitaxel-eluting stents in CTOs. Diffuse atherosclerosis in CTOs can be managed by the DES.

### Dexamethasone-Eluting Stents

Plasma concentrations of intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule-1 [VCAM-1], and E-selectin may increase after percutaneous coronary intervention. A study was carried out to assess whether anti-inflammatory treatment with steroid-eluting stents influenced concentrations of adhesion molecules after this procedure in patients with unstable coronary syndromes (Patti et al. 2005). Consecutive patients with unstable coronary syndromes who were prospectively assigned to undergo implantation of a dexamethasone-eluting stent were compared with a control group of consecutive historical patients who received a similar nondrug eluting stent in the period immediately preceding availability of the steroid-eluting stents. Circulating concentrations of adhesion molecules were measured before the procedure and at 6 and 24 h after implantation. In this study, dexamethasone-eluting stents reduced plasma concentrations of ICAM-1 and VCAM-1 after percutaneous coronary intervention in patients with unstable coronary syndromes compared with historical controls.

The basis for the use of dexamethasone-eluting stents is vascular inflammation in patients with diabetes mellitus (DM), which is a strong predictor of in-stent restenosis. Patients with DM have a higher restenosis rate than nondiabetics following DES procedures. Dexamethasone suppresses local inflammation and consequently neointimal growth with no systemic side effects. Although dexamethasone reduces the restenosis, a clinical study shows that treatment with dexamethasone-eluting stents in patients with DM still has a higher restenosis rate in insulin-dependent compared to non-insulin-dependent DM (van der Hoeven et al. 2008).

## *Novel Technologies for DES*

### Stents for Delivery of Gene Therapy

Stents represent an attractive method for localized gene delivery as they provide a platform for prolonged gene elution and efficient transduction of opposed arterial walls. This gene delivery strategy has the potential to decrease the systemic spread of the viral vectors and hence a reduced host immune response. Both synthetic and naturally occurring stent coatings have the potential to enable prolonged gene elution with no significant adverse reaction.

An adenovirus vector is capable of expressing the tissue inhibitor of metalloproteinase-3 (RAdTIMP-3) and inhibits neointima formation in blood vessels. An eluting stent technology has been developed to deliver RAdTIMP-3 during stenting of pig coronary arteries (Johnson et al. 2005). Binding of virus to and elution from stents and transduction of pig coronary arteries were confirmed using beta-galactosidase as a reporter gene in vitro and in vivo. Deployment of RAdTIMP-3-coated stents increased apoptosis and reduced neointimal cell density, but did not increase inflammation or proliferation. This study demonstrates the feasibility of adenovirus-coated stent technology and indicates the potential of TIMP-3 to produce significant inhibition of in-stent neointima formation.

Gene therapy to treat in-stent restenosis by using gene vector delivery from the metallic stent surfaces has not been previously demonstrated. A study has investigated the hypothesis that metal-bisphosphonate binding can enable site-specific gene vector delivery from metal surfaces. Exposure to aqueous solutions of polyallylamine bisphosphonate (PAA-BP) results in the formation of a monomolecular bisphosphonate layer on metal alloy surfaces (steel, nitinol, and cobalt–chromium), as demonstrated by x-ray photoelectron spectroscopy. Surface-bound PAA-BP enabled adenoviral (Ad) tethering due to covalent thiol-binding of either anti-Ad antibody or a recombinant Ad-receptor protein, D1 (Fishbein et al. 2006). In arterial smooth muscle cell cultures, alloy samples configured with surface-tethered Ad were demonstrated to achieve site-specific transduction with a reporter gene – green fluorescent protein (GFP). Rat carotid stent angioplasties using metal stents exposed to aqueous PAA-BP and derivatized with anti-knob antibody or D1 resulted in extensive localized Ad-GFP expression in the arterial wall. In another study with a model therapeutic vector, Ad-inducible nitric oxide synthase (iNOS) attached to the bisphosphonate-treated metal stent surface via D1, significantly inhibited restenosis. Therefore, effective gene vector delivery from metallic stent surfaces can be achieved by using this approach.

Both adeno-associated virus type 2 (AAV2) and adenovirus, commonly used for gene transfer applications, can achieve prolonged and localized gene delivery to the vessel wall, using stents as delivery platforms. AdβGal and AAV2βGal have been used to coat BiodivYsio stents with matrix HI coating (Abbott Vascular Devices, Galway, Ireland). After balloon injury, external iliac arteries of New Zealand White rabbits were stented and reverse transcription-PCR was used to assess viral spread (Sharif et al. 2006). Expression of LacZ was demonstrated with both vectors at

various time points. No systemic dissemination of virus was seen in any group. It was concluded that adenovirus- and AAV2-coated stents can be used to deliver genes to the blood vessel wall for up to 28 days. The results are being used as a platform to design phase I clinical gene therapy studies in collaboration with partners in the industry. In addition, this mode of gene delivery may also be of relevance to a wide variety of disorders including cancer, as therapeutic products may be delivered at high concentrations specifically to the diseased tissue by this means.

### Stem Cell-Based Stents

Scientists at Sheffield University (Yorkshire, UK) are developing the world's first regenerative stent utilizing stem cells. The body reacts to the presence of the stent as a foreign body. By using the patient's own stem cells to line the stent, one can prevent the reaction. Supportive evidence comes from another study, which showed that stent coating with an integrin-binding cyclic Arg-Gly-Asp peptide may be useful for reducing in-stent restenosis by accelerating endothelialization through recruitment of endothelial progenitor cells (Blindt et al. 2006).

Axordia, in collaboration with Lombard Medical Technologies PLC, is developing a new generation of treatment for coronary artery disease – a regenerative stent. The collaboration between Lombard and Axordia is for a two and a half year project to develop a regenerative stent that encourages rather than restricts local vascular repair. Attaching Axordia's proprietary, stem cell-derived endovascular cells to Lombard Medical's PEP™ programmable polymer coating on the stent surface shall allow the human body to promote controlled vascular repair and heal the damaged coronary artery vessel wall.

The stent shall represent a major step forward into a new format for stent technology where early healing (endothelialization) is promoted rather than using cytotoxic drugs on DES which inhibit growth and can cause late stage thrombotic events. As a result, this stent technology could revolutionize the DES market. A caveat for the use of stem cells for vascular repair is that these cells may be the culprits in the development of restenosis. Scientists at the Weill Cornell Medical College in New York are currently studying how stem cells implant themselves in the wall of arteries and grow out of control following angioplasty and contribute to development of restenosis. The researchers observed that transforming growth factor beta (TGFβ), which stimulates tissue growth, is released in high levels inside the artery following the trauma of angioplasty. This could happen because TGFβ may beckon stem cells to the irritated area to heal the wound, leading to the growth of dense tissue, which blocks the artery. If this mechanism of restenosis is proven, one strategy would be to incorporate a TGFβ antagonist tin the DES to shut off this response.

### Drug-Eluting Stents Coated with Polymer Surfaces

Local drug delivery can be achieved by DES coated with polymer surfaces used for controlled drug release. However, several polymer coatings have shown an induction

of inflammatory response and increased neointima formation. Particle debris shed from a drug-eluting aluminum oxide coating of a stainless steel stent counteracts potential antiproliferative effects of stent-based tacrolimus delivery in a porcine model of restenosis (Kollum et al. 2005). The authors proposed that stent coatings eluting drugs need to be routinely tested for being tightly anchored into the stent surface. Alternatively, omission of any coating used as a drug reservoir may eliminate inflammatory particle debris after placement of drug-eluting stents. The results of this study are different from an earlier study in which ceramic coating of coronary stents with a nanoporous layer of aluminum oxide in combination with tacrolimus resulted in a significant reduction in neointima formation and inflammatory response (Wieneke et al. 2003). The synergistic effects of the ceramic coating and tacrolimus suggest that this new approach may have a high potential to translate into clinical benefit.

### Absorbable DES

Current stent technology is based on the use of permanent implants that remain lifelong in the vessel wall, far beyond the time required for the prosthesis to accomplish its main goals of sealing dissection and preventing wall recoil. With the possibility to implant long vessel segments using antiproliferative drugs to prevent restenosis, the practice of transforming the coronary vessels into stiff tubes with a full metal jacket covering all side branches and being unable to adjust to the long-term wall changes, including wall remodeling with lumen ectasia becomes a serious concern. This is the rationale for the use of first biodegradable stent based on a magnesium alloy that allows controlled corrosion with release to the vessel wall and the blood stream of a natural body component such as magnesium with beneficial antithrombotic, antiarrhythmic, and antiproliferative properties (Di Mario et al. 2004). The authors have conduced animal experiments and have applied this device in 20 PAD patients with stenosis below the knee. There are plans for testing this device in the coronary artery restenosis. The results of these studies will indicate whether the absence of a permanent implant and the antiproliferative properties shown in animals are sufficient to prevent the restenotic process in humans or whether the prosthesis needs to be modified by adding the biodegradable coating with conventional antiproliferative drugs.

### Endeavor DES

Medtronic's Endeavor drug-eluting coronary stent is a drug and device combination incorporating ABT-578 (zotarolimus), a rapamycin analog. It has a cytostatic action. ABT-578 binds to FKBP-12, forming a trimeric complex (ABT-578:FKBP-12) with the protein kinase mTOR inhibiting mTOR's activity. Lack of phosphorylation inhibits transcription leading to the blockage of entry into S phase of cell cycle. The Endeavor system – the first cobalt alloy platform on the drug-eluting stent (DES) market – offers best-in-class deliverability, excellent clinical results, and a strong patient safety profile.

Zotarolimus targets mammalian target of rapamycin, resulting in suppression of cell proliferation. Zotarolimus-coated stent was also developed by Abbott up to


**Fig. 9.3** Medtronic's Endeavor Sprint Zotarolimus-Eluting Coronary Stent System

phase III but did not complete the studies. Abbott noted the removal of the stent from development in lieu of superior efficacy demonstrated by Xience V, another coated stent product that is in development. Medtronic, however, has also developed a zotarolimus-eluting stent (Fig. 9.3).

In 2003, Medtronic started Endeavor De Novo feasibility study, which marked the first human implant of its drug-eluting coronary stent system. The Endeavor feasibility study evaluated the safety and efficacy of Medtronic's DES for the treatment of single de novo lesions in native coronary arteries with a diameter of 3–3.5 mm. It completed clinical trials and CE Mark approval in EU was granted in 2005.

In 2006, impressive long-term clinical results from the ENDEAVOR I and ENDEAVOR II trials were presented at the Paris Course on Revascularization (EuroPCR). Data from the two studies shows that the Endeavor™ DES is continuing to provide significant and sustained efficacy and safety performance over time, with low rates of repeat procedures and no observations of late stent thrombosis. Endeavor™ DES is available in more than 85 countries around the world. In 2007, a FDA advisory committee unanimously recommended the Endeavor® DES for conditional approval as a treatment for coronary artery disease. This action positioned the Endeavor stent to become the first new DES to be introduced in the USA since 2004.

### Bioabsorbable Low-Dose DES

A study of commercially available or investigational paclitaxel-eluting stents as porcine coronary implants has shown a time-dependent phenomenon of vessel wall

toxicity culminating in excessive neointimal formation for paclitaxel elution from a bioabsorbable polymer (Jabara et al. 2006). Paclitaxel toxicity is dose related. Stellium DES (DISA Vascular Ltd) offers a number of clinical advantages over competing products through combining a state-of-the-art stent design, a bioabsorbable polymer and low dose of paclitaxel. The noninflammatory bioresorbable polymer for controlled drug elution enables both early stage elution and late stage elution through gradual but complete polymer erosion. This drug release polymer combines the advantages of the long-term biostability of a proven bare metal stent following complete polymer dissipation within 2 months (obviating the need for long-term antiplatelet medication), and the effective elution properties of a polymer for highly controlled drug diffusion. Stellium is undergoing preclinical studies.

### VAN 10-4 DES

VAN 10-4, a novel small molecule discovered by researchers at University of Strathclyde (Glasgow, Scotland), exerts a marked protection against the development of restenosis when given as a short-term local infusion by localized drug delivery immediately after an angioplasty procedure. It prevents development of neointimal hyperplasia following stent insertion. In vitro studies showed that short-term exposure to VAN 10-4 had no detrimental effect on contractile or relaxant function of blood vessels that were either normal or subjected to injury. VAN 10-4 is significantly more effective as an inhibitor of smooth muscle cell proliferation than paclitaxel. Furthermore, VAN 10-4 was equally effective at inhibiting p42/p44 MAPK activation in human artery cells (derived from atherosclerotic leg arteries) and porcine coronary artery cells, suggesting that its effects on neointimal formation should be evident in the clinical setting.

The use of VAN 10-4 on a DES holds great promise as a superior stent for high risk patients. A VAN 10-4 DES has been remarkably effective in some trials and the drug release characteristics are being optimized. Advantages are:

- Prevention of restenosis in artery stents
- Designed for diabetic and high-risk patients
- Novel antiproliferative mechanism
- No detrimental effects on endothelial function or artery contractility
- Enhanced efficacy and reduced toxicity compared with competitor compounds

## Nanotechnology-Based Stents

### *Restenosis After Percutaneous Coronary Angioplasty*

Restenosis after percutaneous coronary intervention continues to be a serious problem in clinical cardiology. Advances in nanoparticle technology have enabled the delivery of NK911, an antiproliferative drug, selectively to the balloon-injured artery for a longer time (Uwatoku et al. 2003). NK911 is a core-shell nanoparticle

of PEG-based block copolymer encapsulating doxorubicin. It accumulates in vascular lesions with increased permeability. In a balloon injury model of the rat carotid artery, intravenous administration of NK911 significantly inhibited the neointimal formation. The effect of NK911 was due to inhibition of vascular smooth muscle proliferation but not to enhancement of apoptosis or inhibition of inflammatory cell recruitment. NK911 was well tolerated without any adverse systemic effects. These results suggest that nanoparticle technology is a promising and safe approach to target vascular lesions with increased permeability for the prevention of restenosis after balloon injury. Coroxane™ (Abraxis), a nanoparticulate microtubule stabilizer, is in phase II clinical trials in conjunction with angioplasty/stents to prevent arterial restenosis.

Biomedical engineers at Purdue University (Lafayette, IN) have shown that vascular stents used to repair arteries might perform better if their surfaces contained "nano-bumps" that mimic tiny features found in living tissues. The stents, which are made of titanium and other metals, enable the arteries to grow new tissue after vessel-clogging plaque deposits have been removed. A major problem, however, is that the body often perceives the metal devices as foreign invaders, hindering endothelial cells from attaching to the scaffolding and prompting the creation of scar tissue, which can build up inside blood vessels and interfere with blood flow. If a stent does not attach firmly it can become loose, and parts of it will actually break off and go down the bloodstream. There is need for new materials that cause the endothelial cells to attach better to these stents without creating as much dangerous scar tissue. The researchers tested discs of titanium containing surface bumps about as wide as 100 nm. The metals used to make conventional stents have features about ten times larger or none at all. The nanometer-scale bumps mimic surface features of proteins and natural tissues, prompting cells to stick better. Ideally, endothelial cells should quickly attach to stents and form a coating only one cell layer thick. The researchers found that nearly three times as many cells stuck to the discs containing the nanobumps, as compared to ordinary titanium. Further research is planned that will replace the titanium disks with tube-shaped pieces of the nano-featured metal, which will resemble the actual shape of real stents.

Currently available stents have problems with imaging within the stent structure, where potential restenosis can occur. Biophan Technologies Inc has two solutions for stent visibility: a thin-film nanomagnetic particle coating solution and an anti-antenna solution. These solutions enable the noninvasive, MRI-based, imaging of these devices which today can only be accomplished through more complicated invasive procedures. These approaches will become an important part of the rapidly growing worldwide market for stents and vascular implants.

By using antiproliferative compounds that elute from the surface of a stent, the latest generation of stents has enabled a significant reduction in restenosis rates, that is, when there is a renarrowing of the vessel after stent implantation. Nanocarrier-based delivery presents a viable alternative to the current stent-based therapies (Brito and Amiji 2007; Feng et al. 2007; Margolis et al. 2007).

NOLabs is developing NO-eluting nanofibers. One potential application is incorporation into stents for antithrombogenic action. NO has vasodilating action as well, which may be beneficial in ischemic heart disease.

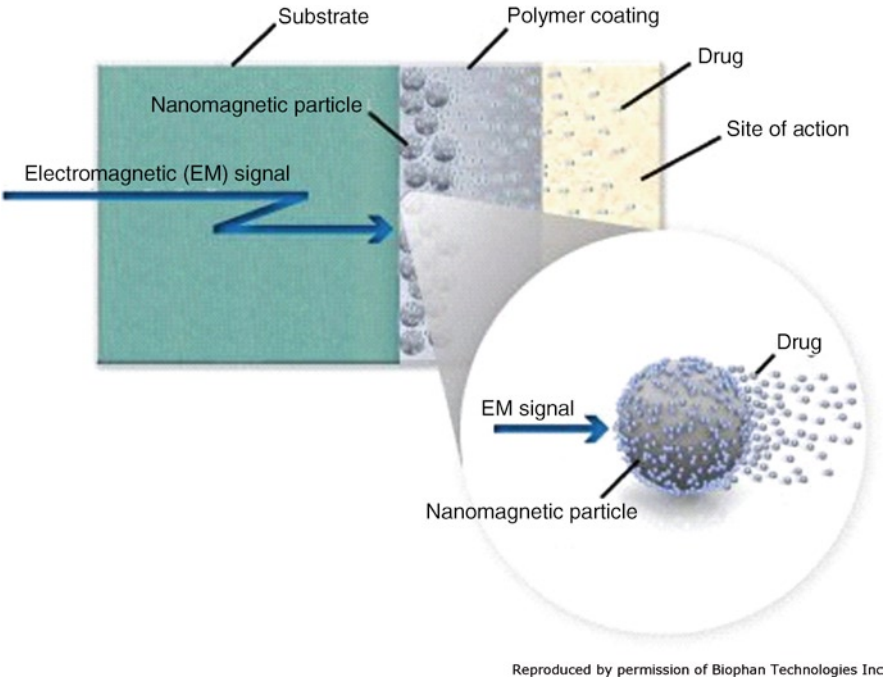


**Fig. 9.4** Magnetic nanoparticle-coated stent

### Drugs Encapsulated in Biodegradable Nanoparticles

Local delivery of antiproliferative drugs encapsulated in biodegradable nanoparticles has shown promise as an experimental strategy for preventing restenosis development. A novel PDGFR β-specific tyrphostin, AGL-2043 (Calbiochem), was formulated in polylactide-based nanoparticles and was administered intraluminally to the wall of balloon-injured rat carotid and stented pig coronary arteries (Banai et al. 2005). The antiproliferative effect of nanoencapsulated tyrphostin was found to be considerably higher than that of surface-adsorbed drug. In the pig model, intramural delivery of AGL-2043 resulted in reduced in-stent neointima formation in the coronary arteries as compared to control, despite similar degrees of wall injury. The results of this study suggest that locally delivered tyrphostin AGL-2043 formulated in biodegradable nanoparticles may be applicable for antirestenotic therapy independent of stent design or type of injury.

### Magnetic Nanoparticle-Coated DES

Biophan Technologies' (Fig. 9.4) drug delivery technology, based on tuning magnetic nanoparticles (MNPs) to resonate at a specific frequency, led to their use for selective control of drug release. This technology which can be used for reloading drug-eluting coatings for surface elution on demand is active in contrast to the

passive drug eluting polymer coatings. It provides a physician better control over the patient's treatment. Currently, many cardiovascular experts predict the next generation of DES will be comprised of a biocompatible, biodegradable, resorbable material with the strength to acutely open and maintain the confirmation of a vessel. The advantage is that they gradually dissolve while delivering the drug. At the end of a predetermined period, nothing is left at the site where it was introduced.

### Magnetic Nanoparticles Encapsulating Paclitaxel Targeted to Stents

Because current DESs lack the capacity for adjustment of the drug dose and release kinetics to the disease status of the treated vessel, attempts have been made to address these limitations by a strategy combining magnetic targeting via a uniform field-induced magnetization effect and a biocompatible MNP formulation designed for efficient entrapment and delivery of paclitaxel (PTX). Magnetic treatment of cultured arterial smooth muscle cells with PTX-loaded MNPs was shown to inhibit cell growth significantly as compared to nonmagnetic conditions (Chorny et al. 2010). Furthermore, significantly higher localization rates of locally delivered MNPs to stented arteries were achieved with uniform field-controlled targeting compared to nonmagnetic controls in the rat carotid stenting model. The arterial tissue levels of stent-targeted MNPs remained four- to tenfold higher in magnetically treated animals versus control over 5 days post delivery. The enhanced retention of MNPs at target sites due to the uniform field-induced magnetization effect resulted in a significant inhibition of in-stent restenosis with a relatively low dose of MNP-encapsulated PTX. This study demonstrates the feasibility of site-specific drug delivery to implanted magnetizable stents by uniform field-controlled targeting of MNPs with efficacy for in-stent restenosis.

### Nanocoated DES

MIV Therapeutics Inc has developed unique coating technologies that utilize hydroxyapatite (HAp) for application on medical devices and drug delivery systems. The lead product in development is a HAp-coated coronary stent with a nanofilm coating. In 2006, the results of an independently conducted 4-week porcine study, performed by the Department of Cardiology, Thoraxcenter, Erasmus University Medical Center in the Netherlands, indicated that three variations of MIV's polymer-free drug-eluting coatings were at least as effective as or better than Cypher (Johnson & Johnson). The study concluded that MIV's HAp coating, with or without drugs, demonstrated highly promising performance. A pilot clinical trial was launched in 2007 and the first HAp-coated was implanted at the Institute Dante Pazzanese of Cardiology in Sao Paulo, Brazil.

ElectroNanoSpray™ formulation technology (Nanocopoeia Inc) produces precise, ultra-pure nanoparticles. Particle sizes can be designed from 2 to 200 nm. The device is capable of applying a coating to the particles in a single process step, producing a drug-loaded core. Competitive processes to produce nanoparticles using wet milling and super critical fluid are inherently limited in their ability to

produce consistently pure particles within a specified size range and distribution. ElectroNanoSpray™ technology provides a novel approach for applying challenging materials to the surfaces of medical devices. This process can generate both single- and multiple-phase coatings and apply these with tight control to small, complex surfaces. ElectroNanospray™ process is being developed for applying nanoparticle-based drug-eluting coatings to coronary stents.

Debiotech SA in collaboration with the Laboratory of Powder Technology at Ecole Polytechnique Fédérale de Lausanne (Lausanne, Switzerland) is developing a new type of structured ceramic coatings for drug-eluting stents and other implants. Ceramics offer unique properties compared to polymers. Polymers dissolve over time and residues provoke inflammation, whereas ceramic is stable and inert when in contact with living tissue. With this coating, one can combine an active release of drug during the first weeks after implantation with the long-term stability of the ceramic. Nanostructured ceramics provide novel properties to biomaterials which are not attainable with other materials. The challenge in this project is to process nano-sized ceramic powders to reach unique surface structures, which show a controlled porosity over a size range of 2,000 times between the smallest and largest pore. Based on results of fundamental research activities in the field of ordered arrangement of nanosized particles at surfaces, the knowledge of processing particles smaller than 10 nm at large scale has been established as a key competence to achieve that goal.

**Nanopores to Enhance Compatibility of DES**

Scientists at the Forschungszentrum Dresden-Rossendorf in Germany have developed an innovative method to create a large number of nanopores on the surface of stainless steel. Bombarding the surface of a stent from all sides with a high dose of noble gas ions generates a scaffold of nanopores in the material below the surface. The desired porosity can be precisely engineered by tuning the ion energy, the flux, and the temperature during the process. A larger amount of the highly effective drugs can be deposited on the enlarged noble metal surface due to this nanoporous structure, which enhances the biocompatibility of the implants in the human body. Thus, this treatment results in the release of drugs over a longer period of time. This method is currently being assessed as a platform technology for the next generation of DES by the Boston Scientific Corporation. The objective of this research collaboration is to further develop this technique for commercialization.

## *The Ideal DES*

The ideal characteristics of a DES for clinical application are:

- Controlled release of the incorporated drug
- Physician control over drug elution according to requirements of an individual patient

- Long-term drug elution and tolerance by the vessel wall
- An easy procedure with a short completion time
- Low cost

All of these requirements are not fulfilled by the available DES. Suggestions for further improvements are made in the section on future prospects at the end of this chapter.

## *Companies Developing Drug-Eluting Stents*

Various companies involved in drug-eluting stents are shown in Table 9.3.

**Table 9.3** Companies involved in drug-eluting stents

| Company | Product | Status |
|---|---|---|
| Abbott vascular devices | Dexamet stent (a drug-eluting stent) elutes dexamethasone into the tissue at the time inflammation and reduces restenosis | Launched in Europe in 2003 |
| | ZoMaxx drug-eluting coronary stent (a unique formulation of phosphorylcholine polymer coating) for the slow, controlled release of ABT-578 – a cytostatic that blocks the function of the cell cycle regulatory protein mTOR | Phase III development discontinued to focus on Xience V |
| | XIENCE V system uses the drug everolimus and MULTI-LINK VISION® coronary stent platform | Launched/Europe Investigational/US |
| Angiotech pharmaceuticals | Polymeric formulations to deliver paclitaxel as a stent coating for restenosis | Phase III |
| Biosensors International | Absorbable DES using Biolimus A9 | Preclinical |
| Boston Scientific | TAXUS Liberté system: Paclitaxel coated stents, which slow microtubule degradation after cell division | Phase III US marketed in other countries |
| Conor Medsystems Inc | CoStar™ (CObalt chromium STent with Antiproliferative for Restenosis) cobalt chromium paclitaxel-eluting stent | Phase II started 2005 |
| Cordis corporation | CYPHER® Sirolimus-eluting Coronary Stent: releases sirolismus, an antirejection drug that limits the overgrowth of normal cells while the artery heals | Marketed |
| Endovasc Ltd | PROStent, comprised of a polymer and PGE-1, slowly releases PGE-1 to prevent restenosis | Completed preclinical |

(continued)

**Table 9.3** (continued)

| Company | Product | Status |
|---|---|---|
| Estacure Inc/MediVas | 17-β-Estradiol drug-eluting stent | Phase II |
| Guidant Corporation | XIENCE™ V, an everolimus-eluting stent bought from Biosensor's International combined with Guidant's cobalt chromium MULTI-LINK VISION® Coronary Stent System | Phase III |
| Implant Sciences Corporation | Stent is fully encapsulated with a drug-eluting microporous polymer sheath, which can be impregnated drugs such as rapamycin or heparin. | Preclinical |
| Medinol Ltd/ARIAD | Stent to deliver ARIAD's mTOR inhibitor, AP23573, to prevent reblockage of injured vessels following stent-assisted angioplasty | In development |
| Medtronic Vascular | Endeavor RESOLUTE zotarolimus-eluting stent system using new biocompatible polymer – BioLinx | CE approval in EU, clinical trials in USA |
| MIV Therapeutics Inc | DES with hydroxyapatite nanofilm coating technology | Clinical trial 2007 |
| REVA Medical Inc | Nitinol Resorbable stent with controlled rate and distribution of drug release. Stent resorbs once the artery has healed | In development |
| Sorin Biomedica Cardio SpA | JUPITER trial using Janus Carbostent based on Carbofilm™ technology and eluting tacrolismus | Phase II |
| Terumo corporation | Nobori™ uses Biolimus A9™ (an analog of sirolimus), is CE approved for marketing in Europe | NOBORI CORE trials |
| XTENT Inc | Custom NX® DES Systems: proprietary stent technology and drug coating, consisting of variable length stents to treat diseased coronary artery lesions | Custom I completed<br>Custom II & III are ongoing |



## *Clinical Trials of Drug-Eluting Stents*

### Measurements Used in Clinical Trials of DES

The safety, efficacy, and performance of drug eluting stents are assessed using certain measurements. Data collected at the time of stent implantation is compared with data collected when a patient is reassessed at follow-up. The time periods for follow-up are usually 6–9 months in pivotal clinical trials for marketing approval in the European Union for CE Mark, and 9 months for clinical trials under an IDE application in the USA conducted to support FDA approval of a PMA application.

Competitors with DES currently being sold in the USA have completed large, prospective, randomized clinical trials that enrolled approximately 1,000 and 1,300 patients each. Measurements used in these clinical trials to evaluate the safety and efficacy of DES include the following:

*Late loss of lumen diameter*. The clinical trials of currently FDA approved drug eluting stents demonstrated in-stent loss of 0.17–0.39 mm, and in-segment loss of 0.23–0.24 mm at 8–9 months.

*Binary restenosis rate*. This is defined as the percentage of patients that have a greater than 50% reduction in the lumen diameter from the time of stent implantation to the time of follow-up. The measurement may either be in-stent or in-segment. The clinical trials of currently FDA approved drug eluting stents demonstrated in-stent restenosis of 3.2–5.5%, and in-segment restenosis of 7.9–8.9% at 8–9 months.

*Percent volume obstruction*. This is defined as the volume of the lumen in the stent that is occupied by restenotic tissue. The percent volume lumen obstruction is measured using intravascular ultrasound. The clinical trials of currently FDA approved DES demonstrated volume obstruction of 3.1–12.2% at 8–9 months.

*Target lesion revascularization (TLR) rate*. This is defined as the percentage of patients at follow-up who required another coronary intervention, such as balloon angioplasty or a CABG procedure, to treat the same lesion in the artery, within the stent or within 5 mm on either side of the stent. The clinical trials of currently FDA approved drug eluting stents demonstrated TLR rates of 3.0–3.9% at 8–9 months.

### TAXUS Paclitaxel-Eluting Stents

The polymer carrier technology in the TAXUS drug-eluting stent consists of a thermoplastic elastomer poly(styrene-b-isobutylene-b-styrene) (SIBS) with microphase-separated morphology resulting in optimal properties for a drug-delivery stent coating. Comprehensive physical characterization of the stent coatings and cast film formulations showed that paclitaxel exists primarily as discrete nanoparticles embedded in the SIBS matrix (Ranade et al. 2004).

The TAXUS Liberté coronary stent system will be the next generation to Boston Scientific's market-leading paclitaxel-eluting coronary stent system, TAXUS® Express2™. The Liberté stent features the Veriflex™ stent design, a highly flexible cell geometry with thin struts and uniform cell distribution. This new platform has been designed to offer improved deliverability and conformability in challenging anatomy. It also features the enhanced TrakTip™ catheter tip, mounted on the Maverick2™ delivery catheter, to provide better lesion crossability. In addition, TrakTip has a low lesion-entry profile, also intended to further improve crossability.

A randomized triple-blind study has evaluated paclitaxel-eluting stents (PES) for the treatment of restenosis (Park et al. 2003). Coronary stents significantly improved the angiographic outcome 6 months after percutaneous transluminal coronary intervention by reducing neointimal hyperplasia after stent placement, resulting

in increased vessel diameter, reduced stenosis, and reduced binary restenosis at follow-up. Paclitaxel had a safety profile similar to that of the control stent when used with conventional antiplatelet therapy.

The available data from the TAXUS trials have demonstrated that implantation of the slow rate-release paclitaxel-eluting TAXUS stent is safe and, in terms of restenosis, markedly superior to bare metal stenting for the treatment of de novo lesions <28 mm in length in arteries 2.5–3.75 mm in diameter (Halkin and Stone 2004). In order to overcome the uncertainty concerning results of PES implantation in very high-risk patients and lesions, a prospective multicenter registry, the Taxus in Real-life Usage Evaluation (TRUE) Study was designed (Biondi-Zoccai et al. 2007).

Boston Scientific Corporation is enrolling patients in the world's largest drug-eluting stent registry – TAXUS OLYMPIA registry. The registry plans to enroll more than 30,000 patients at more than 600 centers in the USA, Europe, and other international locations. The registry is designed to collect and analyze "real-world" clinical outcomes data for Boston Scientific's next-generation TAXUS® Liberté™ paclitaxel-eluting stent system in the treatment of patients with CAD. The registry will evaluate a variety of safety and performance measures, including the rate of repeat procedures (target lesion revascularization), major adverse cardiac events such as heart attack and death, and in-stent thrombosis. Additionally, sub-analyses on complex patient groups such as diabetics, patients with multivessel disease, in-stent restenosis, or a prior history of heart attack will be performed. The registry will enroll patients in five phases, corresponding to the commercial introduction of the TAXUS Liberté system in different regions of the world. In 2006, the enrollment had exceeded 13,000 patients in the registry and the final two-phase US enrollment was completed at end of 2006 when BSC presented data on its 7,000-patient ARRIVE I and II registries of real-world patients – including those with complex lesions – to a special FDA panel. The data showed that the TAXUS® paclitaxel-eluting coronary stent provides substantial benefits in keeping arteries open and avoiding repeat procedures for patients with complex coronary artery disease, at no higher risk than alternative cardiovascular treatments.

One clinical trial of TAXUS has explored the effect of celecoxib, based on cell and animal studies, which have shown that celecoxib inhibits Akt stimulation and restenosis. The COREA-TAXUS (Effect of Celecoxib on Restenosis after Coronary Angioplasty with Taxus stent) trial was performed in subjects with angina or a positive-stress test receiving paclitaxel-eluting stents (Koo et al. 2007). The primary end point at 6 m was the in-stent, late luminal loss, which was 0.49 mm in the celecoxib-treated group; less than the 0.75 mm in the group not treated with celecoxib. The rate of revascularization of the target lesion was lower in celecoxib-treated subjects (5%) than in the untreated subjects (15%).

### XIENCE™ V Everolimus-Eluting Coronary Stent

The angiographic findings of the first human randomized trial of the everolimus-eluting stent (EES) as compared to bare metal stent for the treatment of noncomplex coronary showed that at 6-month follow-up of 42 patients, EES had a lower in-stent late lumen loss and in-segment diameter stenoses (Costa et al. 2005). There was no in-stent restenosis with EES; however, one focal distal edge restenosis was

present. There was one in-stent and one in-segment (proximal edge) restenosis in the metallic stent group. There was no stent thrombosis or aneurysm formation at follow-up in either group. Another study found that between 2 and 4 years after implantation of sirolimus-eluting stent, peristent tissue shrank with a concomitant increase in echogenicity as demonstrated by serial quantitative intravascular ultrasound and computer-assisted grayscale value analysis (Aoki et al. 2005). These findings suggest that the neointima does not significantly change from 2 to 4 years and that the biological phenomenon of a delayed healing response starts to subside. Although several studies established the superiority of coronary EES over paclitaxel-eluting stent system (PES) with respect to angiographic findings, these trials were not powered for superiority in clinical end points.

XIENCE™ V is Abbott Vascular's EES using MULTI-LINK VISION® Coronary Stent System platform is currently an investigational device in the USA and Japan. The six studies involving 8,000 patients for evaluating this system for the treatment of coronary artery disease are:

SPIRIT I – First human study showed positive results through 2 years with no major adverse cardiac events for patients with previously untreated native coronary artery lesions.

SPIRIT II – 6 m results of the trial conducted in Europe and Asia-Pacific demonstrated superiority of the XIENCE V stent system over the TAXUS® PES with respect to the study's primary endpoint, which was angiographic in-stent late loss at 6 m.

SPIRIT III – This large-scale randomized pivotal clinical trial in 2006 compared the XIENCE V system to the TAXUS stent system in the USA and Japan.

SPIRIT IV – This trial evaluated the safety and efficacy of the XIENCE V system for the treatment of coronary artery disease in a more complex patient population in the USA; 3,687 patients were randomly assigned at 66 US sites to receive EES or PES without routine follow-up angiography (Stone et al. 2010). The primary end point was the 1 year composite rate of target-lesion failure (defined as cardiac death, target-vessel myocardial infarction, or target-lesion revascularization). EES, as compared with PES, resulted in reduced rates of target-lesion failure at 1 year, results that were consistent in all patients except those with diabetes, in whom the results were nonsignificantly different.

SPIRIT V – This study will provide additional clinical experience with the XIENCE V system throughout Europe, Asia-Pacific, and Canada in real-world patients. One subset of the study is dedicated to diabetic patients.

SPIRIT WOMEN – The world's first DES trial to study only women will evaluate the characteristics of women undergoing stent implantation and performance of XIENCE V in those patients in Europe, Asia-Pacific, Canada, and Latin America.

### COSTAR II Clinical Trial

COSTAR™ (CObalt chromium STent with Antiproliferative for Restenosis) is developed by Conor Medsystems Inc and is being studied in COSTAR II

US pivotal clinical trial, which is designed to randomize approximately 1,700 patients at up to 75 US sites and 15 international sites. Conor expects the data from this trial to support its application for US regulatory approval of its CoStar cobalt chromium paclitaxel-eluting stent. The design of CoStar stent appears to have several potential advantages as compared to conventional drug eluting stents, including a low profile for ease of navigation and placement in smaller vessels, the ability to control the release kinetics of the drug, and the use of a bioresorbable polymer that leaves no residual polymer or drug on the stent. The COSTAR II trial is a randomized, single-blind, noninferiority study comparing Conor's CoStar stent with Boston Scientific's TAXUS® Express2 drug-eluting stent in the treatment of de novo lesions in patients with single or multivessel coronary artery disease. The CoStar stent is loaded with a dose of 10 mcg of paclitaxel per 17 mm stent using a bioresorbable polymer, a formulation determined to be efficacious during the company's European clinical studies. Patients will be asymmetrically randomized between CoStar and the control stent with clinical follow-up at 30 days and 8 months. In addition, a 350-patient subset will undergo follow-up angiography at 9 months. Enrollment is anticipated to be completed in approximately 6–9 months. The primary endpoint for the study will be major adverse cardiac events (MACE) at 8 months, defined as a composite of target vessel revascularization (TVR), myocardial infarction, and cardiac-related death. Other endpoints include target lesion revascularization (TLR), binary restenosis, and in-segment and in-stent late loss as measured by angiography.

### Endeavor RESOLUTE Zotarolimus-Eluting Stent System

Current DESs do not meet all the requirements of physicians who deal with the most challenging clinical cases, such as patients with diabetes. This newest DES innovation from Medtronic, zotarolimus-eluting stent system, leverages the strengths of the Endeavor stent and introduces a proprietary, new biocompatible polymer called BioLinx, which is designed to help match the duration of drug delivery with the longer healing duration often experienced by patients with complex medical conditions. BioLinx is a noninflammatory polymer blend that mimics the structure of a cell membrane to maintain biocompatibility. Its outer surface is hydrophilic, which leads to high biocompatibility with the body, while the interior of the polymer is hydrophobic, which helps to precisely control the drug release. As a result, it offers uniform drug dispersion, sustained elution, and the opportunity for multiple drug platforms.

Positive preliminary results were reported in 2006 from the Medtronic RESOLUTE clinical trial, a first-in-man study evaluating the new Endeavor RESOLUTE zotarolimus-eluting stent system. Four-month angiographic and clinical results reinforce that zotarolimus continues to be a very potent drug in preventing restenosis, even in challenging patient populations. The trial has a primary endpoint of late lumen loss (in-stent) at 9 months and customary angiographic, intravascular ultrasound (IVUS) and clinical secondary endpoints. Thirty-day clinical results for 130 patients showed a MACE rate of 3.8%, with zero TLR and no stent thrombosis. In 30 patients with 4-month angiographic follow-up, in-stent late

loss was 0.12 mm and in-segment late loss was 0.05 mm. Both in-stent and in-segment binary restenosis were zero, and IVUS results showed neointimal volume obstruction of 2.2% at 4 months. Stent device and lesion success was 100%, which means that physicians were successful in placing the assigned stents in the proper location with few complications.

In 2007, a FDA advisory committee unanimously recommended the Endeavor® DES for conditional approval as a treatment for coronary artery disease. This action positions the Endeavor stent to become the first new DES to be introduced in the USA since 2004.

## CUSTOM I Clinical Trial

The XTENT® Custom NX® stent's proprietary modular design consists of multiple 6 mm cobalt chromium segments that are interdigitated. The stent's interdigitation is designed to enable separation at each 6 mm segment, and increase flexibility during delivery and after implantation, while maintaining the radial strength necessary to hold the artery open across multiple segments. The drug coating consists of Biolimus A9® (Occam International), an anti-inflammatory drug that is a derivative of rapamycin, and a PolyLactic Acid (PLA) coating, a biodegradable polymer used to release the drug over time. Following the placement of a guidewire, the XTENT Custom NX® DES System is inserted into the femoral artery and the catheter is maneuvered to the site of the target lesion. Opaque markers on the balloon inflation lumen and the sheath allow for visual assessment of stent length and location relative to the target lesion.

CUSTOM I clinical trial was designed to evaluate the preliminary safety and efficacy of in situ customization using XTENT proprietary stent technology and drug coating, consisting of a 36 mm stent to treat diseased coronary artery lesions in 2.6–3.1 mm diameter arteries. The trial has been completed and results are due to be announced. Custom II and III are ongoing and further trials are planned.

## NOBORI CORE Trial

Nobori™ DES (Terumo Corporation), which uses Biolimus A9™ (an analog of sirolimus), is CE approved for marketing in Europe. It was tested in a program with two phases: NOBORI CORE and NOBORI Pharmacokinetics study. In randomized studies versus Taxus Express® and Taxus Libertè®, the Nobori™ stent proved to be equal and even superior in efficacy endpoints such as late loss, with an exceptionally low frequency of adverse cardiac events and no stent thrombosis up to 1 year in phase I and 9 months in phase II. In NOBORI CORE, a comparative study versus Cypher® stent, Nobori™ also showed excellent performance with very low rate of adverse cardiac events. The overall restenosis rate in all NOBORI trials was as low as 0.5% and no late stent thrombosis was recorded in any of the trials. Terumo plans additional clinical activities with the Nobori™ stent as part of a comprehensive program to characterize the stent's long-term safety and efficacy in a variety of patient populations. The program will enroll more than 5,000 patients in randomized trials and post-marketing registry in Europe, Asia, New Zealand, and Africa.

### LEADERS Trial

LEADERS (Limus Eluted from A Durable versus ERodable Stent coating) was a head-to-head comparison of Biosensors Biolimus-eluting DES with an abluminal biodegradable polymer and Johnson & Johnson's Cypher sirolimus-eluting DES with a durable polymer. Results released in 2008, showed that efficacy and safety of both DES was equal.

This multicenter European study randomized 1,707 patients eligible for percutaneous coronary intervention (PCI) for symptomatic coronary disease to receive either Biolimus-eluting DES or a Cypher DES. In total, 2,472 coronary lesions were treated. Inclusion criteria were broad, reflecting routine clinical practice, without limitations regarding type of coronary vessel, lesion length, or number of treated lesions. Patient conditions known as "off-label indications," including acute coronary syndromes, saphenous vein grafts, and previously treated lesions were also included in the trial. The primary endpoint of the study was noninferiority of the composite of cardiac death, myocardial infarction, and clinically driven target vessel revascularization at 9 months follow-up. In addition, 25% of all patients were randomly assigned to undergo angiographic follow-up at 9 months. The principal endpoint of the prespecified angiographic subgroup was in-stent percent diameter stenosis at 9 months.

During the first 9 months, 9.2% of patients receiving the Biosensors DES, and 10.5% of patients given the Cypher DES experienced a clinical adverse event that could be included in the primary composite endpoint, thus demonstrating that the Biosensors stent was not inferior to the Cypher stent. A favorable trend toward the Biosensors stent was nonsignificant at the 9 months follow-up endpoint. As anticipated, clinical event rates were higher in LEADERS compared with previous DES trials performed in patients with only on-label indications, because the LEADERS trial design permitted inclusion of any patient eligible for PCI. As a result, rates of death, myocardial infarction, and stent thrombosis were similar for both stent types, but were 2.6% higher when compared to the earlier, less inclusive trials. In the angiographic subgroup, there were no significant differences at 9 months between the in-stent percent diameter stenosis observed in the two patient groups, but there was a nonsignificant trend favoring the Biosensors stent. The results from LEADERS are very significant as they demonstrate for the first time that a drug-eluting stent with an abluminal biodegradable polymer is as safe and effective at 9 months as a conventional DES with a durable polymer, considered to be the most effective, under conditions which resemble those of routine clinical practice. Longer-term follow-up of the patients in LEADERS or studies of a similar nature are now needed to confirm the theoretical advantage of the abluminal biodegradable polymer in terms of reduced risk of late thrombosis.

### Comparison of DES in Clinical Trials

A systematic review was reported of the randomized clinical trials that have evaluated the efficacy and safety of DESs (Eisenberg and Konnyu 2006). A total of

28 randomized clinical trials were identified: 21 comparing a DES (sirolimus, paclitaxel, ABT-578, actinomycin, everolimus, or 7-hexanoyltaxol) with a BMS and 7 comparing a DES with another DES (sirolimus vs paclitaxel). Early sirolimus and polymeric paclitaxel studies in low-risk populations demonstrated marked reductions in restenosis according to angiographic and clinical parameters, compared with bare metal stents (BMSs). These promising findings led to the more recent evaluations of DESs in higher risk patients in controlled and head-to-head comparisons. In these subsequent trials, sirolimus and paclitaxel DESs continued to exceed the therapeutic potential of BMSs, with a slight but consistent angiographic advantage being observed with the sirolimus DESs.

In a multicenter, noninferiority trial with minimal exclusion criteria, 2,292 patients were randomly assigned to undergo treatment with coronary stents releasing either zotarolimus or everolimus (Serruys et al. 2010a). The primary end point was target-lesion failure, defined as a composite of death from cardiac causes, any myocardial infarction (not clearly attributable to a nontarget vessel), or clinically indicated target-lesion revascularization within 12 months. The secondary angiographic end point was the extent of in-stent stenosis at 13 months. The new-generation zotarolimus-eluting stent was found to be as safe and effective as the everolimus-eluting stent in a group of patients for whom the procedure was considered to be predominantly off-label.

## *Comparison of DES with Competing Technologies*

### DES Versus Coronary Artery Bypass Graft

While drug eluting stenting represents a major breakthrough in interventional cardiology, there is still a significant need for coronary artery bypass graft (CABG) surgery in the treatment of patients with blocked coronary arteries. Although drug-coated stents are a definite improvement over bare stenting, restenosis has not been eliminated and they cannot be used in all patients. Failure of DES to prevent restenosis may be due to technical factors. Stent underexpansion is a significant cause of failure after sirolimus-eluting stent implantation treatment of in-stent restenosis (Fujii et al. 2004). Furthermore, better long-term outcomes are anticipated with the introduction of internal mammary artery grafts, less invasive surgical procedures, smaller incisions, and off-pump surgery in the near future. The main drawback of CABG was vein graft failure and vein graft atherosclerosis. The rate of vein graft failure can be decreased somewhat by technical refinements, platelet inhibitors, and statins. No one knows the very long-term effects of DES even though the patency of the vessel is maintained. The question whether such arteries may develop an aneurysm cannot be answered as yet. For extensive and multiple obstructions in the coronary arteries, CABG may still be a better option. For patients with multivessel disease, CABG continues to be associated with lower mortality rates than does treatment with DES, and is also associated with lower rates myocardial infarction and repeat revascularization (Hannan et al. 2008).

In 2009, Boston Scientific reported 2-year data from its SYNTAX clinical trial comparing percutaneous coronary intervention using the Taxus® Express® Paclitaxel-Eluting Coronary Stent System to CABG surgery. The overall results demonstrated no statistically significant difference between the two procedures in the composite safety endpoint (all-cause death, stroke, and myocardial infarction).

### DES Versus Bare Metal Stents

A prospective, placebo-controlled, double-blind, multicenter randomized trial has shown that in comparison with a BMS, implantation of the PES in a patient population with complex lesions effectively reduces clinical and angiographic restenosis (Stone et al. 2005). According to another study, DES is a safe and effective treatment for MI, provoking no increase in complications when compared to conventional stenting and cutting the risk of arterial renarrowing by 90% (Cheneau et al. 2005). This fills an important information gap in the treatment of high-risk patients. Until now, patients with MI have been excluded from studies of DES. Physicians, however, often use drugs and devices that have been approved in one group of patients to extend treatment to a new group of patients. There is increasing use of DES as the primary treatment for acute MI. In this study, patients treated with the Cypher stent (Cordis Corporation) were compared to MI patients who had been treated with a BES in the preceding 5 years. Treatment of MI patients with the DES was found to be safe. There was no difference between the two groups in procedural complications. Another important finding was that no patient developed subacute thrombosis, a serious complication, in the DES within a month of treatment. This is especially reassuring, given that MIs are themselves often caused by a thrombus that blocks the artery following rupture of an inflamed plaque. The study also showed that DES accomplished their primary purpose, preventing the need for a repeat procedure to treat renarrowing in the stented artery. At 6-month follow-up, only 1% of patients treated with the DES required a new procedure to reopen the artery, as compared to more than 10% of patients treated with the bare metal stents. The investigators of this study are now considering a trial of head-to-head comparison of two types of DES in the treatment of heart attack: the Cypher stent and its competitor, the Taxus stent (Boston Scientific), which is coated with a different anti-restenosis drug – paclitaxel.

One study compared the efficacy of the sirolimus-eluting stent (SES), the paclitaxel-eluting stent (PES), and the BMS for long coronary lesions (Kim et al. 2006). The study concluded that use of DESs for long coronary lesions appears to be safe and more effective than the use of BMSs in terms of restenosis and adverse clinical events. SES use was associated with lower late luminal loss and a lower angiographic restenosis rate compared with PES use. A study has pooled analysis of data from several double-blind trials in which a large number of patients were randomly assigned to receive either PES or SES or BMS and analyzed the major clinical end points of the trials (Stone et al. 2007). Results showed that stent thrombosis after 1 year was more common with both SES and PES than with BMS. Both DES were associated with a marked reduction in target-lesion revascularization.

There were no significant differences in the cumulative rates of death or myocardial infarction at 4 years.

Another study investigated the effect of SES implantation in a high-risk population, i.e., patients who had diabetes and small coronary vessel disease by analyzing outcomes of the subset of patients who had diabetes and were enrolled in the SES-SMART, a randomized trial that compared the results of implantation of SESs and BMSs in small coronary arteries (Ortolani et al. 2005). The use of SESs was associated with approximately 60% decreases in the relative incidence of in-segment angiographic restenosis and in-segment late loss. Angiographic patterns of restenosis were more favorable in the SES group. SES implantation was associated with a 15% absolute decrease in adverse clinical events. In patients who had insulin-dependent diabetes mellitus, SESs showed a high in-segment restenosis rate (40%) that was principally due to persistent restenosis. The study concluded that in diabetics with small coronary arteries, SES implantation significantly reduces the incidence of the 8-month angiographic restenosis rate compared with BMS. A pooled analysis was performed of 1,748 patients in four randomized trials evaluating the safety of SES as compared with BMS (Spaulding et al. 2007). Results show that the survival rate at 4 years was 93.3% in the sirolimus-stent group, as compared with 94.6% in the BMS group. In the 428 patients with diabetes, a significant difference in the survival rate was observed in favor of the BMS group over the SES group. The lower survival rate among patients with diabetes who were treated with SES was due to increased numbers of deaths from both cardiovascular and noncardiovascular causes. No difference in survival rate was detected among the patients without diabetes. Rates of myocardial infarction and stent thrombosis were similar in the two groups. In conclusion, no significant differences were found between the two treatments in rates of death, myocardial infarction, or stent thrombosis.

In one study, DES implantation for de novo coronary lesions in patients with significant chronic kidney disease resulted in a lower 1-year incidence of clinical and angiographic restenosis compared to those with BMS implantation (Jeong et al. 2008). Review of a large series of patients revealed that the use of DES in the Swedish "real world" is effective in reducing the clinically driven restenosis rate, when compared with patients with BMS treatment (Jensen et al. 2006). In the angiographic follow-up, the average late loss was as low as observed in recent randomized multicenter trials. A study evaluated 6,033 patients treated with DES and 13,738 patients treated with BMS in 2003 and 2004, using data from the Swedish Coronary Angiography and Angioplasty Registry (Lagerqvist et al. 2007). The outcome analysis covering a period of up to 3 years was based on 1,424 deaths and 2,463 myocardial infarctions and was adjusted for differences in baseline characteristics. Results showed that the two study groups did not differ significantly in the composite of death and myocardial infarction during 3 years of follow-up. At 6 months, there was a trend toward a lower unadjusted event rate in patients with DES than in those with BMS, with 13.4 fewer such events per 1,000 patients. However, after 6 months, patients with DES had a significantly higher event rate, with 12.7 more events per 1,000 patients per year. At 3 years, mortality was significantly higher in patients with DES, and from 6 months to 3 years, the adjusted relative risk for death in this group

was 1.32. It was concluded that DES were associated with an increased rate of death, as compared with BMS. This trend appeared after 6 months, when the risk of death was 0.5 percentage point higher and a composite of death or myocardial infarction was 0.5–1.0 percentage point higher per year. The long-term safety of DES needs to be ascertained in large, randomized trials.

Diabetes mellitus is a major risk factor for restenosis in patients undergoing percutaneous coronary intervention. Randomized controlled trials comparing DES with BMS showed a marked decrease in in-stent restenosis and target lesion revascularization with DES in the total patient population enrolled in the studies, including patients with diabetes. Review of clinical trials comparing DES with BMS in patients with diabetes shows that DES use is associated with a marked decrease in in-stent restenosis and target lesion revascularization (Boyden et al. 2007). The analysis supports the current widespread use of DES in these high-risk patients.

Most of the DES studies have been done in coronary arteries. Internal mammary arteries (IMAs) and veins are also used for revascularization of the myocardium in myocardial ischemia. One study examined the safety and efficacy of DES for the treatment of lesions in the IMA conduits and compared the outcomes with those from BMS (Buch et al. 2006). DES implantation into IMAs appears safe and is associated with low rates of recurrences. These results may support expansion of use of DES for the management of IMA stenotic lesions.

A study at the Wake Forest University School of Medicine (Winston-Salem, NC) investigated the outcome of BMS placed in 1,164 consecutive patients in the year before the introduction of DES, which were subsequently placed in 1,285 consecutive comparable patients (Applegate et al. 2007). At 9 months, target vessel revascularization and death were lower in the DES group than in the BMS group. In conclusion, in this single-center observational study, use of DES in consecutive unselected patients, most of whom would not have been eligible for inclusion in the randomized trials of DES versus BMS, was associated with lower acute myocardial infarction and death rates than in a comparable group of patients treated with BMS in mid-term (9-month) follow-up. In another study the widespread adoption of DES into routine practice was associated with a decline in the need for repeat revascularization procedures and had similar 2-year risks for death or ST-elevation myocardial infarction to bare-metal stents (Malenka et al. 2008). With the current data DES might still have an advantage over BMS in the treatment of myocardial infarction with ST-segment elevation with the vastly improved target vessel revascularization rate by continuing long-term dual antiplatelet therapy, which has been shown to be safe and effective in these patients (Yeter et al. 2009). In patients with ST-segment elevation myocardial infarction who were undergoing primary percutaneous coronary intervention, implantation of paclitaxel-eluting stents, as compared BMS, significantly reduced angiographic evidence of restenosis or recurrent ischemia necessitating repeat revascularization procedures and no safety concerns were apparent at 1 year (Stone et al. 2009).

In a large study conducted at Harvard Medical School on patients presenting with acute myocardial infarction, treatment with DES was associated with decreased 2-year mortality rates and a reduction in the need for repeat revascularization

procedures as compared with treatment with BMS (Mauri et al. 2008). Another study has evaluated 47,967 patients in Sweden who received a coronary stent and were entered into the Swedish Coronary Angiography and Angioplasty Registry between 2003 and 2006 and for whom complete follow-up data were available for 1–5 years (James et al. 2009). In the primary analysis, patients who received one DES (10,294 patients) were compared with those who received one BMS (18,659), after adjustment for differences in clinical characteristics of the patients and characteristics of the vessels and lesions. As compared with BMS, DES are associated with a similar long-term incidence of death or myocardial infarction and provide a clinically important decrease in the rate of restenosis among high-risk patients.

In a randomized study, patients requiring stenting of large (3 mm diameter) coronary arteries, no significant differences were found among sirolimus-eluting, everolimus-eluting, and BMS with respect to the rate of death or myocardial infarction (Kaiser et al. 2010). With the two DES', similar reductions in rates of target-vessel revascularization were seen.

### Guidelines for DES Versus BMS

The Task Force on Practice Guidelines of American College of Cardiology/American Heart Association has published the 2007 update of the2005 guidelines for percutaneous coronary intervention (King et al. 2007). The following are relevant to DES versus BMS:

1. A DES should be considered as an alternative to a BMS in those patients for whom clinical trials indicate a favorable effectiveness/safety profile. (Level of Evidence: A).
2. Before implanting a DES, the interventional cardiologist should discuss with the patient the need for and duration of DAT and confirm the patient's ability to comply with the recommended therapy for DES. (Level of Evidence: B).
3. In patients who are undergoing preparation for PCI and are likely to require invasive or surgical procedures for which DAT must be interrupted during the next 12 months, consideration should be given to implantation of a BMS or performance of balloon angioplasty with a provisional stent implantation instead of the routine use of a DES. (Level of Evidence: C).

### DES Versus BMS for Off-Label Indications

Off-label use of stenting is defined as application in restenotic lesions, lesions in a bypass graft, left main coronary artery disease, or ostial, bifurcated, or totally occluded lesions, as well as use in patients with a reference-vessel diameter of less than 2.5 mm or greater than 3.75 mm or a lesion length of more than 30 mm. Both BMS and DES have been used for off-label indications and one study has compared the use of BMS with DES in off-label indications in patients who were followed for 1 year for the occurrence of cardiovascular events and death (Marroquin et al. 2008).

Off-label use occurred in 54.7% of all patients with BMS and 48.7% of patients with DES. As compared with patients with BMS, patients with DES had a higher prevalence of diabetes, hypertension, renal disease, previous percutaneous coronary intervention and CABG, and multivessel coronary artery disease. One year after intervention, however, there were no significant differences in the adjusted risk of death or myocardial infarction in patients with DES as compared with those with BMS, whereas the risk of repeat revascularization was significantly lower among patients with DES. It was concluded that among patients with off-label indications, the use of DES was not associated with an increased risk of death or myocardial infarction but was associated with a lower rate of repeat revascularization at 1 year, as compared with BMS. These findings support the use of DES for off-label indications.

### Role of DES in Cases of Bare-Metal In-Stent Restenosis

Although there is clinical evidence that DES are associated with better results than other treatments for in-stent restenosis, they are not yet approved for this indication. Meta-analysis of randomized trials may yield more precise estimates of treatment effects and enable a rapid adoption of effective treatments in clinical practice. One such study showed that the risk of target lesion revascularization and that of angiographic restenosis were markedly lower in patients treated with DES (Dibra et al. 2007). Using DES to replace bare metal stents that have become blocked may be the best solution to reopening cardiac arteries and keeping them open. In fact, replacing bare metal stents with drug-coated stents cuts the risk of this reclosure, called restenosis, by 65%, compared with either balloon angioplasty or radiation treatment.

There were no differences between patients treated with DES and those treated with other techniques with respect to the composite of death or myocardial infarction. DES are markedly superior to conventional techniques (balloon angioplasty and vascular brachytherapy) and should be considered as first-line treatment for patients with bare-metal in-stent restenosis. The finding of this study echoes the general consensus among cardiologists.

### DES Versus Balloon Catheter Coated with Paclitaxel

A randomized, double-blind, multicenter trial compared the effects of a balloon catheter coated with paclitaxel with those of an uncoated balloon catheter in coronary angioplasty patients with in-stent restenosis (Scheller et al. 2006). The primary end point was late luminal loss as seen on angiography. Secondary end points included the rates of restenosis (a binary variable) and major adverse cardiac events. Results showed that treatment of coronary in-stent restenosis with paclitaxel-coated balloon catheters significantly reduced the incidence of restenosis. These data suggest that the inhibition of restenosis by local drug delivery may not require stent implantation and sustained drug release at the site of injury.

A prospective, randomized, multicenter, 2-armed phase II pilot study was conducted in Europe to compare the combination of a paclitaxel-coated balloon + BMS

(Coroflex® DEBlue) with sirolismus-eluting Cypher stent in the treatment of de novo stenosis in native coronary arteries. Results released in 2009 showed that Coroflex did not meet the noninferiority criteria versus Cypher. However, late lumen loss was comparable to published data on paclitaxel-eluting stents. It was concluded that further design improvement is needed to improve this new approach.

### DES Versus Intracoronary Radiation Therapy for Recurrent Stenosis

Recurrent in-stent restenosis (ISR) following intracoronary radiation therapy (IRT) continues to be a therapeutic challenge. A study has evaluated the clinical outcomes of patients who were treated with DES implantation versus repeat IRT for recurrent ISR after brachytherapy failure (Chu et al. 2005). Patients who were previously treated with brachytherapy for ISR and presented with angina and recurrence of angiographic restenosis were evaluated for treatment with either DES – either sirolimus-eluting stents (SES) or paclitaxel-eluting stents (PES) or PCI and repeat radiation (gamma or beta radiation). The two groups had similar baseline clinical and angiographic characteristics. The in-hospital outcomes were similar between both groups. There was a trend toward more target vessel revascularization, major adverse cardiac events in the DES group. In addition, the patients in the DES group had a significantly lower survival rate compared to those in the repeat IRT group. For patients who have recurrent ISR following IRT, either DES implantation or repeat radiation is safe and is associated with excellent immediate outcomes. Yet, at long-term follow-up, repeat IRT is associated with less recurrences and need for repeat revascularization when compared to DES implantation. Therefore, repeat IRT should be considered as an option for this difficult patient. Another randomized comparison between IRT and implantation of paclitaxel-eluting stents for the treatment of diffuse ISR revealed a significant reduction of binary restenosis in the Taxus-Express2 arm of the study, and there was no difference in clinical outcome after implantation of paclitaxel-eluting stents for the treatment of diffuse ISR when compared to IRT (Schukro et al. 2006).

A prospective, multicenter, randomized trial showed that sirolimus-eluting stents result in superior clinical and angiographic outcomes compared with vascular brachytherapy (VBT) for the treatment of restenosis within a BMS (Holmes et al. 2006). A prospective, multicenter, randomized trial has shown that treatment of bare-metal in-stent restenotic lesions with paclitaxel-eluting stents rather than angioplasty followed by VBT reduces clinical and angiographic restenosis at 9 months and improves event-free survival (Stone et al. 2006).

## *Cost-Effectiveness of DES*

In the Basel stent cost-effectiveness trial (BASKET), patients were randomized to one of two DES (Cypher and Taxus) or to a cobalt-chromium-based BMS and followed up for 6 months for occurrence of major adverse cardiac events and costs (Kaiser et al. 2005). The primary endpoint was cost-effectiveness after 6 months.

Total costs at 6 months were higher with DES ($12,560) than with BMS ($11,482); higher stent costs of DES were not compensated for by lower follow-up costs. Incremental cost-effectiveness ratio of DES compared with BMS to avoid one major event was $21,812 and costs per quality-adjusted life-year gained were more than $59,554. Subgroup analyses showed that use of DES was more cost-effective for elderly patients in specific high-risk groups. Thus, in a real-world setting, use of DES in all patients is less cost-effective than in studies with selected patients. It was concluded that use of these stents could be restricted to patients in high-risk groups.

An independent analysis of a clinical trial comparing the cost-effectiveness of the CYPHER® Sirolimus-eluting Coronary Stent versus bypass surgery, presented in suggests that treatment with the CYPHER® Stent offers a potential cost saving over bypass surgery. Treatment of multivessel coronary artery disease with the CYPHER® Stent was found to be substantially more cost-effective than bypass surgery both during initial hospitalization and at the 1-year follow-up. Initial hospitalization costs, accounting for physician fees, room and ancillary charges, and repeat procedures, were $7,700 less (29%) for CYPHER® Stent than for CABG, at $26,419 and $34,119, respectively. Total 1-year costs, when adjusted for patient differences in extent of coronary artery disease and diabetes, were $6,487 less (21%) for CYPHER® Stent than for CABG, at $30,388 and $36,875 respectively.

## *Safety Issues of DES*

### Adverse Reactions to DES

DES reactions may be due to the drugs released or the material of the stent. A small percentage of patients are allergic to the polymer DES and a handful have died from the reactions, according to an adverse-event records review initiated by cardiologists from the Health Science Center of the University of Texas (Houston, TX). The prevalence of allergic reactions to DES is unknown, although the allergy risk to the vast majority of patients is small. A skin test has been devised for detecting polymer hypersensitivity. The patients in question are not allergic to the stent itself, but to the polymer coating the metal used to hold the drugs for 30-day release. A next generation of DES is needed to deliver drugs without polymers. In some patients, the stents can cause allergic reactions that can have serious consequences (Nebeker et al. 2006). For this study, RADAR (Research on Adverse Drug/Device Events And Reports) investigators from ten centers around the USA reviewed 5,783 reports available from 2003 to 2004 for hypersensitivity-like reactions associated with DES. From these reports, researchers identified 17 cases of hypersensitivity reactions that were classified as probably or certainly caused the stent, four of which resulted in death. Symptoms included rash, difficulty in breathing, hives, itching, and fevers. They also concluded that the polymer coating on the stent itself is the most probable cause of hypersensitivity in the majority of cases rather than the medications the stent is coated with. Another concern is that in many instances,

blame for the allergic symptoms is placed on other medications that treat their heart conditions, such as Plavix® and antiplatelet agents, and these are discontinued. Premature discontinuation of Plavix® can increase the risk of dangerous blood clots. The study also concludes that further research is warranted to better understand this risk and to develop a skin test to identify people who might be at high risk for hypersensitivity to DES. A new drug delivery technology, using a porous stent surface, may offer desirable drug elution properties without the use of polymers, and may translate into an improved safety profile for the next-generation DESs (Tsujino et al. 2007).

### Endothelial Vascular Dysfunction due to Sirolismus

Despite excellent antirestenotic capacity, sirolimus-eluting stents have been found to trigger coronary endothelial dysfunction and impaired reendothelialization. A study has sought to analyze mechanisms that mediate vascular dysfunction induced by sirolimus (Jabs et al. 2008). To mimic the continuous sirolimus exposure of a stented vessel, Wistar rats underwent drug infusion with an osmotic pump for 7 days. Results showed that sirolimus causes marked vascular dysfunction and nitrate resistance after continuous treatment for 7 days. This impaired vasorelaxation may, in part, be induced by upregulated mitochondrial superoxide release as well as by an upregulation of NADPH oxidase-driven superoxide production. Both processes could contribute to endothelial dysfunction observed after coronary vascular interventions with sirolimus-coated stents.

### Risk of Clotting with DES

In a long-term follow-up study, the incidence of late stent thrombosis after DES implantation was 0.6%, similar to that for BMS. The predictors of stent thrombosis were premature antiplatelet therapy interruption, primary stenting in acute myocardial infarction, and total stent length (Park et al. 2006). A hierarchical classification of stent thrombosis set by the Academic Research Consortium across randomized trials was applied to analysis of pooled 4 years of follow-up data on patients treated with SES, PES, and BMS (Mauri et al. 2007). The cumulative incidence of stent thrombosis according to the original protocol definitions was 1.2% in the SES versus 0.6% in the BMS group and 1.3% in the PES group versus 0.8% in the BMS group. The incidence of definite or probable stent thrombosis as defined by the ARC was 1.5% in the SES group versus 1.7% in the BMS group and 1.8% in the PES group versus 1.4% in the BMS group. The incidence of definite or probable events occurring 1–4 years after implantation was 0.9% in the SES group versus 0.4% in the BMS group and 0.9% in the PES versus 0.6% in the BMS group. It was concluded that the incidence of stent thrombosis did not differ significantly between patients with DES and those with BMS in randomized clinical trials, although the power to detect small differences in rates was limited.

The clotting risk may be a small percentage, but with so many of the DES now in use, at least 2,160 extra deaths occur each year in the USA alone. Clotting has

always posed a risk during and shortly after stenting. But the clot risk virtually disappears for BMS after a few months, allowing patients to stop taking anticlotting drugs. New data suggest that the risks of thrombosis do not disappear for the DES that now dominate the market. The exact reasons are not known, but many investigators believe the stents can create a home for clots, because either their drugs or the stents' plastic surfaces inhibit complete healing of the vessel wall.

Unlike restenosis, stent thrombosis is a potentially life-threatening complication of coronary stents. Nearly half of the episodes occurred >30 days (late stent thrombosis) and almost half of these resulted in death. The strongest independent predictor of stent thrombosis was premature discontinuation of dual antiplatelet therapy (Iakovou et al. 2005). Another article concluded: "it makes little clinical, economic, or common sense to forsake a therapy that works well for most patients (BMS) in favor of a costly new therapy (DES) that has no effect on important clinical outcomes but increases the risk for stent thrombosis, a life-threatening complication (Tung et al. 2006)."

Advocates of DES point to evidence that many clotting cases involve stents that were not fitted snugly to the arterial wall. That misstep can be eliminated if doctors monitor their work during the procedure with new ultrasound scanning technology as well as the standard angiograms.

Risk of DES thrombosis is particularly high after discontinuation of antiplatelet therapy, increasing the risk to as high as 30-fold in some studies. The American Heart Association, American College of Cardiology, Society for Cardiovascular Angiography and Interventions, American College of Surgeons, and American Dental Association have issued a joint advisory recommending adherence to current guidelines for patients undergoing DES implantation. This will usually require patients taking both aspirin and a thienopyridine (most commonly clopidogrel) for periods of up to at least 1 year after stenting and aspirin indefinitely. Patients should not discontinue either aspirin or the thienopyridine within the first year without consulting their treating cardiologist. The following are recommendations to eliminate early discontinuation of dual antiplatelet therapy:

1. Before implanting a stent, the physician should discuss the need for antiplatelet therapy with the patient. In patients not expected to comply with 12 months of thienopyridine therapy, for whatever reasons, a BMS should be strongly considered.
2. In patients who are likely to require surgery within 12 months of receiving a stent, a BMS or balloon angioplasty with a provisional stent should be considered instead of routinely using a drug-eluting stent.
3. Healthcare professionals must make a greater effort to educate patients before hospital discharge about the reasons for prescribing thienopyridines and for taking the dual antiplatelet therapy, as well as the risks for stopping it early.
4. Patients should be specifically instructed before hospital discharge to contact their cardiologist before stopping any antiplatelet therapy, even if instructed to do so by another healthcare provider.
5. Healthcare providers who perform invasive or surgical procedures should be made aware of the potentially catastrophic risks of prematurely stopping

thienopyridine therapy, and should contact the patient's cardiologist to discuss optimal patient management.
6. Elective procedures that carry a risk of bleeding should be delayed until a month after the patient has completed an appropriate course of thienopyridine therapy, which is ideally 12 months after receiving a drug-eluting stent in patients who are not at high risk of bleeding, and at least 1 month after a bare metal stent.
7. For patients who receive a drug-eluting stent and who must have procedures that mandate stopping thienopyridine therapy, aspirin should be continued if at all possible, and the thienopyridine restarted as soon as possible after the procedure (due to concerns about late stent thrombosis).
8. The healthcare industry, insurers, congress, and pharmaceutical industry should insure that issues such as drug cost do not cause patients to prematurely discontinue thienopyridine therapy and incur catastrophic cardiovascular complications.

### Clopidogrel Use and Long-Term Outcomes of Patients Receiving DES

Clopidogrel (sold as Plavix) can be used along with aspirin for 3–6 months following stent placement to prevent thrombosis, but the drug is not free of risks. It can cause thrombotic thrombocytopenic purpura, a rare disorder of the blood coagulation system. Also, clinical trials have revealed that use of clopidogrel can, in rare cases, increase risk of hemorrhage or lead to decreased levels of white blood cells, and, more commonly, cause stomach pain, gastrointestinal disturbances, headaches, and dizziness. Patients taking clopidogrel must stop taking the drug if they are scheduled to undergo surgery or some other type of invasive medical procedure that might lead to bleeding.

Recent studies of DES suggest that current antiplatelet regimens may not be sufficient to prevent late stent thrombosis. An observational study of clopidogrel use and long-term clinical outcomes of patients receiving DES and BMS for treatment of coronary artery disease has concluded that the extended use of clopidogrel in patients with DES may be associated with a reduced risk for death or myocardial infarction (Eisenstein et al. 2007). Heart patients who have received drug-coated stents to hold open an artery and who stop taking the drug clopidogrel to reduce blood clotting may face more than double the risk of death or heart attack than patients who continue on the drug. However, the appropriate duration for clopidogrel administration can only be determined within the context of a large-scale randomized clinical trial.

### Effect of Blood Clot on Release of Drug from DES

Although a DES delivers potent compounds directly to arterial segments, it can become clot laden when deployed. The affect of thrombi on paclitaxel elution and arterial uptake has been studied. Blood cells bind and retain paclitaxel such that levels in clot increase linearly with red cell fraction. At physiological hematocrit, clot retains three times the amount of paclitaxel in surrounding solutions. Computational models predict that the potential of thrombus to absorb,

retain, and release drug or to act as a barrier to drug delivery depends on clot geometry and strut position in clot relative to the vessel wall. Clot between artery and stent can reduce uptake tenfold, whereas clot overlying the stent can shield drug from washout, increasing uptake. These assumptions were confirmed and predictions were validated in a novel rat model that introduces thrombosis within stented aortas where nonocclusive thrombus acts as capacitive space for drug and shifts drug levels to decrease tissue uptake twofold (Hwang et al. 2005). Thus, the thrombus formed on stents creates large variations in drug uptake and can act to either increase or decrease wall deposition according to the clot and stent geometry. Arterial deposition of drug from stents deployed in clots will be highly variable and unpredictable unless the clot can be adequately controlled or removed.

### Use of Magnetized Cell Lining to Prevent Clotting of DES

A novel approach to prevent blood clotting in DES is based on magnetizing endothelial progenitor cells from the patient's blood, which are quickly drawn to magnetically coated stents (Pislaru et al. 2006). Cultured porcine endothelial cells, extracted from blood, were labeled with endocytosed superparamagnetic iron oxide microspheres. After the stent was implanted, the iron-tagged cells were introduced back into the blood vessel to test how well the magnetized stents captured the cells. Because the endothelial progenitor cells naturally counteract blood clot formation, their swift magnetically guided arrival to the stent may reduce the chances of blood clot formation by lining the site fully and quickly. Results from preclinical testing are encouraging. There was a 6- to 30-fold improvement in the magnetized stents' performance in capturing the healing endothelial cells, compared to the standard stents' ability to do so.

### Long-Term Safety Studies of DES

The trend in medicine in the USA and Europe is evidence-based medicine, which is use of current best evidence in making decisions about the care of individual patients. Successful implementation of evidence-based medicine in the development of high-risk medical devices requires a methodical scientific approach. In case of DESs, even when research evidence is available from randomized controlled studies, it can be difficult to translate clinical evidence into regulatory and clinical decisions (O'Dwyer 2004).

One study has investigated the frequency of off-label and untested use, 1-year repeat target vessel revascularization, and composite of death, myocardial infarction, or stent thrombosis at in-hospital follow-up and during 1 year of follow-up (Beohar et al. 2007). The results show that in contemporary US practice, off-label and untested use of DES is common. Compared with standard use, relative early safety is lower with off-label use, and the long-term effectiveness is lower with both off-label and untested use. However, the absolute event rates remain low.

In contrast to this, another study showed that compared with on-label use, off-label use of drug-eluting stents is associated with a higher rate of adverse outcomes during the index admission and at 1 year (Win et al. 2007). Stent thrombosis occurred predominantly in patients who underwent off-label drug-eluting stent implantation.

By reducing restenosis, the DES have saved tens of thousands of patients from the risks of follow-up stenting procedures or bypass surgery. But it would take a study involving tens of thousands of patients and as many as 5 years to produce highly reliable data on whether those benefits offset the risks of the blood clotting now associated with the DES. Therefore, long-term safety data are essential for DESs. After a DES gains market approval, the FDA requires quarterly report of details of follow-up on each stent in addition to the usual adverse event reporting. Follow-up studies lasting at least 5 years must be performed and manufacturers must set up a national registry to follow at least 2,000 patients for 1 year. Boston Scientific is taking a lead in this matter by TAXUS study.

As DES implantation becomes more liberal leading to an extensive use of this technology, the problem of restenosis in DES will become more common. However, little is yet known about optimal treatment of in-stent restenosis following DES implantation. Future research is needed to determine if these patients should be treated with the same drug-eluting stent, with a different drug-eluting stent, or with increased doses.

In one study of patients, endothelium-dependent vasomotion of a coronary segment 15 mm in length, starting 2 mm distal to the SES, was assessed with quantitative coronary angiography immediately after the procedure and at 6 months follow-up, after intracoronary infusion of acetylcholine (Hofma et al. 2005). Intravascular ultrasound was performed and coronary flow reserve assessed in all patients. Results of this study indicate that SES implantation may have an adverse effect on local endothelium-dependent vasomotor responses compared with bare metal stent implantation at 6 months. Long-term clinical consequences of this observation are still unknown.

A study in Canada showed that the 2-year rate of target-vessel revascularization was significantly lower among patients who received DES than among those who received BMS (Tu et al. 2007). DES were associated with significant reductions in the rate of target-vessel revascularization among patients with two or three risk factors for restenosis (i.e., presence of diabetes, small vessels, and long lesions) but not among lower-risk patients. The 3-year mortality rate was significantly higher in the BMS group than in the DES group. It was concluded that DES are effective in reducing the need for target-vessel revascularization in patients at highest risk for restenosis, without a significantly increased rate of death or myocardial infarction.

## Regulatory Issues of DES

In 2006, the FDA provided the following information in response to inquiries on the agency's position on adverse events related to coronary DES. A summary is as follows:

- FDA has been monitoring coronary DES closely since they came on the US market in 2003 and 2004, and will continue to do so.
- New data were released recently that suggest a small but significant increased risk of stent thrombosis in patients who have DES. The agency is keenly interested in this issue because of the potential for serious harm to patients – even though stent thrombosis occurs at low rates.
- While the new data are of interest to FDA and raise important questions, it does not have enough information yet to draw conclusions. It is unclear, for example, what causes DES thrombosis, how often it occurs, under what circumstances it occurs, or what the risk of occurrence is in a given patient.
- To better understand this issue, FDA met with the two manufacturers of these products in recent months to discuss any information they might have pertaining to this issue and get their perspective. In addition, it plans to convene a public panel meeting of outside scientific experts in the near future to assist us in a thorough review of all the data and make recommendations about what actions may be appropriate, such as possible labeling changes or additional studies.
- At this time, FDA believes that coronary drug-eluting stents remain safe and effective when used for the FDA-approved indications. These devices have significantly reduced the need for a second surgery to treat restenosis for thousands of patients each year.

There are reports of subacute thrombosis associated with the stents and associated patient deaths. Taxus Express was associated with failure of the stent delivery balloon to deflate after the stent was inserted; these were associated with serious injuries and death. Boston Scientific traced the problem to a manufacturing error and corrected it. Although the adverse events are rare, risks have to be balanced against benefits in DES. Obviously, such challenges will continue to occur with breakthrough technologies and devices. The FDA perspective at that time was summarized in a publication (Muni and Gross 2004). DES have been evaluated in patients previously only considered for surgical intervention. Assessment of DES in these complicated patient populations can lead to challenges in trial design, but the FDA has considered alternative clinical trial designs and statistical analysis plans (Boam 2006). Other complex issues associated with DES include duration of clinical trials to determine safety, and the appropriate dose and duration of concomitant antiplatelet therapy.

In the light of the published studies suggesting that DES may pose a risk of thrombosis that was not observed during premarket testing, the FDA convened a meeting of its Circulatory System Devices Advisory Panel in 2006 to examine the safety of these devices. The FDA will carefully consider the information and views presented at the meeting in deciding on future actions. The panel agreed, and the FDA concurs, that when DES are used for their approved indications, the risk of thrombosis does not outweigh their advantages over BMS in reducing the rate of repeated revascularization (Farb and Boam 2007). But the panel also concluded that, as compared with on-label use, off-label use is associated with increased risks of both early and late stent thrombosis, as well as death or myocardial infarction. The panel concluded that

safety and effectiveness of DES as compared with those of alternative treatments deserve continued study. The lessons that have been learned and the answers to the remaining questions will facilitate the development and review of future DES.

In 2008, the FDA issued draft guidelines to aid the development, testing, and manufacture of DES. Concerned about clot formation in some patients years after implantation, the agency has monitored the devices closely over the past several years. Two coronary DES are currently FDA-approved: (1) Cypher (Cordis) and (2) TAXUS® (Boston Scientific). The guidance document also assesses the toxicity of the drug used to coat the stent, both on its own and as part of the complete product. These stents combine device and drug technology and, as such, the document contains expertise and input from two agency centers – the Center for Devices and Radiological Health and the Center for Drug Evaluation and Research.

The relevance of clinical trial data to the "real world" of clinical practice is questionable. After EU and USA launches of Cypher and Taxus, several single and multicenter registries were set up to assess DES effectiveness in a broader range of patient subgroups. These include e-CYPHER, an internet-based European post-marketing surveillance targeting 15,000 patients who have received a least one Cypher stent.

Some studies have compared DESs using different drugs. A systematic review and meta-analysis was performed using indirect comparisons and the evidence indicates that stents eluting sirolimus are superior to paclitaxel eluting stents in patients without diabetes but not in patients with diabetes (Stettler et al. 2006). Thus variable factors such as background disease of the patient may make direct comparison of various stents difficult. The FDA acknowledges that DES are complex products to produce, and it believes that through interaction with the FDA during development, difficulties with test methodologies, animal studies, and clinical trial designs can be addressed. The future of DES likely involves new stent and carrier materials, including biodegradable materials and new drugs and biologicals. The FDA anticipates continued collaboration with physicians, manufacturers, academic institutions, and professional societies.

## Future Prospects for Treatment of Restenosis by DES

### *Future Role of DES in Management of Cardiovascular Diseases*

Use of DES needs to be evaluated in the context of constant reassessment of treatment of cardiovascular diseases. A randomized trial involving patients who had objective evidence of myocardial ischemia and significant coronary artery disease, percutaneous coronary intervention did not reduce the risk of death, myocardial infarction, or other major cardiovascular events when added to optimal medical therapy (Boden et al. 2007). It means that many heart patients routinely implanted with stents to open arteries gain no lasting benefit compared with those treated just with drugs. Although patients with stents to prop open coronary blood vessels, in addition to being treated with statins and other heart drugs in a 5-year trial, had

better blood flow to the heart than patients treated only with drugs, they did not live longer or suffer fewer heart attacks. The investigators also found that the stents were highly successful at improving blood flow and relieving symptoms, including chest pain and shortness of breath, but that the advantage disappeared over time.

Other studies have shown that angioplasty and stenting can save lives when used in heart attacks. The procedure is also recommended for many patients whose symptoms of poor blood flow to the heart are not relieved by rest or drugs. Although modern drugs slowed the progression of coronary artery disease, stenting is better for relieving symptoms, so both should be available. For such patients, the controversy is not whether to use stents in addition to drugs but whether stents are used in too many seriously ill patients who might live longer with coronary bypass surgery.

It is not simply a question of DES versus CABG. Another option is hybrid coronary revascularization, which is a combination of minimally invasive coronary artery bypass grafting and percutaneous coronary intervention in patients with multivessel coronary artery disease (Friedrich and Bonatti 2007). The surgical part of the procedure can be performed in a totally endoscopic fashion instead of by a mini-incision approach, and catheter-based intervention includes the use of DES. Whereas during early development, staged approaches were taken, simultaneous interventions have become feasible. Hybrid procedures are an attractive option for high-risk patients or for patients who seek a less traumatic revascularization option.

To resolve the controversy about DES and to provide additional evidence for the regulators, the following recommendations are made to encourage evidence-based patient management (Kaul et al. 2007):

1. An emphasis on medical therapies with proven long-term benefit.
2. The use of kinetic modeling to estimate long-term outcomes of therapies based on the available near-term data.
3. The restructuring of reimbursement incentives to encourage the use of evidence-based clinical management strategies.

## *Stent Cost and Marketing Strategies*

DES will continue to be used in clinical practice despite their high initial cost (about three times that of a BMS) because the cost is likely to be compensated for by the reduced need for repeat revascularization. Angioplasty with stenting generally costs $25,000 and up. The latest DESs cost $2,200 apiece and are especially effective at preserving the channel created by angioplasty (see Chap. 6). The costs of stents should go down as more manufacturers enter the market, especially from Asia. DES are proving to be useful for this indication or until an efficient and reliable antiproliferative genetic therapy becomes available.

Approximately, 60–75% of the millions of drug eluting stents inserted globally are not licensed. A complex interplay of factors may explain the high DES use beyond an evidence base. These include collaboration between the cardiology

community and device industry to push new technology, an interventionist community that romanticizes new technology, and overdependence on interventionism when treating coronary syndromes.

## *Improvements in Stent Technology*

The future lies in delivery of multiple drugs at timed intervals through new stent designs. Gene eluting stents are undergoing experimental and clinical trials and it is expected that gene eluting stents alone or in conjunction with other DES will be available in the near future. There are still concerns about a more prolonged risk of stent thrombosis. Although all agree on the need for a longer duration of dual antiplatelet therapy in patients treated with DES, its optimal length is still to be defined. Because polymers used for stent coating are often seen at the origin of the compromised long-term safety of DES, new technologies able to avoid permanent polymers may offer a valuable alternative (Iijima et al. 2006).

With the rapid ascent of stent-based drug elution in the treatment of vascular diseases, important issues regarding drug distribution and targeting need to be addressed. Transport forces and drug physicochemical properties contribute in varying degrees to vascular drug distribution. Device proximity to target tissue thus does not assure adequate distribution, and a detailed grasp of local pharmacokinetics is a prerequisite for the rational design of drug delivery systems.

Technical advances are providing the development of improved materials for coating of DES. Nanomaterials are the most prominent among these. Another important development is the bioabsorbable stent.

### Bioabsorbable Stent

Stents do not need to be permanent. If an artery stays open for 6 months after being unclogged, it is essentially healed. Leaving it any longer would create complications such as blood clots. Fully bioabsorbable scaffolds are a novel approach that provides transient vessel support with drug delivery capability without the long-term limitations of the metallic DES. The technology has the potential to overcome many of the safety concerns associated with metallic DES, and possibly even convey further clinical benefit. Although the technology is still in its infancy, several devices have been tested in clinical trials and the initial results have been very promising (Onuma et al. 2011).

The bioabsorbable stent is designed to dissolve once it finishes the job. It is made of the same kind of material as certain dissolvable stitches, but designed to last longer. It is coated with a drug to prevent reclogging. After the drug has permeated the artery walls, the stent starts dissolving. Animal studies suggest the body completes its breakdown of the device in about 2 years. The first generation of the bioresorbable everolimus drug-eluting vascular scaffold showed signs of shrinkage at 6 months, which largely contributed to late luminal loss, although it was less than that observed with BMS. To maintain the mechanical integrity of the device up to

6 months, the scaffold design and manufacturing process of its polymer were modified, which have substantially improved the medium-term performance of this new generation of drug-eluting scaffold to become comparable to those of current drug eluting stents (Serruys et al. 2010b). Clinical trials with such devices are under way and the initial results are encouraging. The advantages of biodegradable polymers include high drug loading capacity, controlled long-term drug release, and full degradation of the polymer over a certain time resulting in full release of the drug during a well-controlled time interval.

### *DES Versus Drug-Eluting Balloons*

Non-stent-based methods for local drug delivery enable restenosis inhibition without the need for stent implantation. They do not permanently change the structure of the vessel, are repeatable, and seems to be applicable where DES provide insufficient protection. Preclinical data indicate that short exposure of the vessel wall to a lipophilic inhibitor of cell proliferation is sufficient for preventing restenosis. Initial evidence to this effect emerged from an investigation of paclitaxel embedded in a matrix that enhances the solubility and release of the agent from the balloon coating as well as its transfer to the vessel wall (Schnorr et al. 2010). Further corroborating data from preclinical and clinical studies demonstrating a reduction in late lumen loss and lower restenosis rates led to the market introduction of a variety of paclitaxel-coated angioplasty balloons. The effectiveness of restenosis inhibition is not determined by the active agent alone. Other factors that are crucial for the effectiveness and safety of drug-coated balloons (DEBs) for angioplasty are the formulation containing the agent and the coating technique.

A preclinical study was done to optimize the use of DEB DIOR (second generation) by measurements of tissue and plasma paclitaxel concentrations in porcine coronary artery overstretch and to prove efficacy in inhibition of neointimal growth without complementary use of stent (Pósa et al. 2010). Paclitaxel penetrated up to 2 mm tissue deepness. Measurable plasma paclitaxel level was found only after 60 s balloon inflation time. At follow-up, the dilated arterial segment neointimal area and maximal neointimal thickness were significantly smaller with DIOR versus uncoated balloon use. Fluorescence images of DIOR showed a homogenous distribution of the drug on the vessel, in contrast with DES. The study concluded that using the DIOR(secondgeneration) DEB, a maximal balloon inflation time of 30–45 s is optimal, reducing effectively the neointimal hyperplasia.

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# Chapter 10
# Personalized Cardiology

## Introduction to Personalized Medicine

Personalized medicine simply means the prescription of specific treatments and therapeutics best suited for an individual (Jain 2009). It is also referred to as individualized or individual-based therapy. Personalized medicine is based on the idea of using a patient's genotype as a factor in deciding on the treatment options, but other factors are also taken into consideration. Genomic/proteomic technologies have facilitated the development of personalized medicines but other technologies are also contributing to this effort. Application of the principles of personalized medicine to management of cardiovascular diseases is referred to as "personalized cardiology."

The constantly growing volume of available data will require an organized interpretation of variations in DNA and mRNA as well as proteins, both on the individual and population level. A five-step strategy can be followed when trying to identify genes and gene products involved in differential responses to cardiovascular drugs (Siest et al. 2007):

1. Pharmacokinetic-related genes and phenotypes
2. Pharmacodynamic targets, genes, and products
3. Cardiovascular diseases and risks depending on specific or large metabolic cycles
4. Physiological variations of previously identified genes and proteins
5. Environmental influences on them

Gene–environment interactions are important in many human diseases. Genetic analysis of thousands of transcript abundance traits in human primary endothelial cell lines in response to proinflammatory oxidized phospholipids implicated in cardiovascular disease revealed that approximately one third of most regulated transcripts, showed evidence of gene–environment interactions (Romanoski et al. 2010). The interactions resulted primarily from effects of distal-, trans-acting loci, but a striking example of a local gene–environment interactions interaction was also observed for FGD6. Some of the distal interactions were validated by siRNA knockdown experiments. These findings are consistent with the possibility that gene–environment interactions are responsible, in part, for the failure of association studies to fully explain common disease variation.

K.K. Jain, *Applications of Biotechnology in Cardiovascular Therapeutics*,
DOI 10.1007/978-1-61779-240-3_10, 

## Role of Diagnostics in Personalized Management of Cardiovascular Disease

### *Testing in Coronary Heart Disease*

In ischemic heart disease, the patient's arteries have narrowed and the heart cannot pump normally because blood flow (and thus oxygen) is often restricted to the heart muscle. In nonischemic forms of the disease, the heart cannot pump normally because the heart muscle has often enlarged for other reasons, such as physical deformity or alcohol abuse. Both conditions can lead to cardiac arrest or more gradual heart failure as the muscle weakens over time. Differentiation between the two types is important for planning the management. The next step is to develop a test that can be used in a clinical setting. Ischemic patients need to be monitored more closely in case they develop drug resistance and require surgery to unblock clogged arteries. Knowing which patients to treat and how closely to monitor them could significantly improve how well physicians manage the disease and, consequently, improve health outcomes.

Lp-PLA2 (lipoprotein-associated phospholipase A2) is an enzyme that is implicated in the vascular inflammatory pathway that leads to plaque formation and atherosclerosis. Previous hypotheses on the cause of coronary heart disease focused around lipid accumulation within the arterial walls. Increasing evidence now suggests that atherosclerosis is largely an inflammatory disease. The MONICA (MONItoring of trends and determinants in CArdiovascular disease) study showed a statistically significant relationship between elevated Lp-PLA2 and the risk of a coronary event (Koenig et al. 2004). Among individuals in the MONICA population, each standard deviation increase in Lp-PLA2 levels resulted in a 37% increase in the risk of a coronary event. This study also showed that Lp-PLA2 and C-reactive protein, a marker of inflammation, may be additive in their ability to predict risk of coronary heart disease.

Routine cholesterol tests account for only about 50% of the predictability in heart disease risk. A test based on Vertical Auto Profile (VAP, Atherotech Inc) technology for density gradient ultracentrifugation directly measures the cholesterol content of all lipids, components, and subclasses. VAP is an expanded cholesterol profile that provides direct, detailed measurements of cholesterol, or lipid, subclasses which play important roles in the development of cardiovascular disease. The test identifies twice the number of people at risk for heart disease than traditional cholesterol tests developed in the 1970s. Measurements obtained using. VAP test also provide physicians with a foundation from which to develop individualized treatment plans while continuing to track patients' progress in battling heart disease.

### *SNP Genotyping in Cardiovascular Disorders*

Scientists at the Joslin Diabetes Center (Boston, MA) have invented diagnostic methods to detect an individual's susceptibility to developing cardiovascular

disease by analysis of specific SNPs within the receptor gene that correlate to the disease risk. Two specific SNPs were analyzed and found to correlate to risk of coronary artery disease (CAD) in two specific populations. Minor allele homozygotes for one of the SNPs had more than a twofold increase in CAD risk across both populations. Homozygotes for a particular haplotype of the other SNP were 1.7-fold more likely to have had a myocardial infarction. In addition, homozygotes for the first SNP showed 30% lower levels of mRNA for the receptor than other subjects. The invention therefore features methods of diagnosing or detecting susceptibility toward cardiovascular disease by typing specific SNPs in the genome of an individual.

Common SNPs at 18 loci are reproducibly associated with concentrations of LDL cholesterol, HDL cholesterol, and/or triglycerides. Six of these loci are new, and of these two are associated with LDL cholesterol (1p13 near CELSR2, PSRC1 and SORT1 and 19p13 near CILP2 and PBX4), one with HDL cholesterol (1q42 in GALNT2), and five with triglycerides (7q11 near TBL2 and MLXIPL, 8q24 near TRIB1, 1q42 in GALNT2, 19p13 near CILP2 and PBX4, and 1p31 near ANGPTL3). At 1p13, the LDL-associated SNP is also strongly correlated with CELSR2, PSRC1, and SORT1 transcript levels in human liver, and a proxy for this SNP has been shown to affect risk for coronary artery disease. A genotype score of nine validated SNPs that are associated with modulation in levels of LDL or HDL cholesterol is an independent risk factor for incident cardiovascular disease (Kathiresan et al. 2008). The score does not improve risk discrimination but modestly improves clinical risk reclassification for individual subjects beyond standard clinical factors.

## *Cardiovascular Disorders with a Genetic Component*

Genetic testing can be effectively used to distinguish between heart failure patients who suffer from ischemic or nonischemic forms of the disease. Johns Hopkins scientists have used groupings or clusters of a patient's gene expression to compare to a diseased "test" set that identifies the cause of heart failure. Using a biostatistical technique of prediction analysis, the investigators have identified the 90 genes that best distinguished the two kinds of heart failure. The large number of genes used also improved accuracy of the test. Results showed the test profile to be highly accurate, with 90% specificity. The findings could, if confirmed and adapted to a standardized and affordable test format, someday aid physicians in the diagnosis of heart failure and help determine which kind of therapy is best to use for the condition.

Several cardiovascular diseases are recognized to have a genetic component; indeed, a family history of heart disease has always attracted the physician's attention. In recent years, molecular genetics has contributed to the development of molecular cardiology, opening up some new pathways to the diagnosis, prevention, and treatment of some cardiovascular diseases. Genetic approaches have succeeded in defining the molecular basis of an increasing array of heart diseases, such as hypertrophic cardiomyopathy and the long-QT syndrome (Brugada Syndrome),

a potentially fatal cardiac disorder associated with serious arrhythmias. Some of the genes that cause cardiovascular diseases are shown in Table 10.1.

Long Q-T syndrome is an inherited form of ventricular arrhythmia in which the interval between the Q and the T waves is longer than normal. This disease reflects a defect in the electrical properties of the cardiac muscle, which predisposes the patient to life-threatening ventricular fibrillation after stress. Five genes have been identified where the mutations are associated with this disorder. These genes encode cardiac potassium ion channels and support the hypothesis that the LQT syndrome results from delayed myocellular repolarization. The diagnosis of long QT syndrome and other channelopathies by an electrocardiogram is often difficult and may be missed, which leaves a patient at risk for sudden cardiac death. FAMILION™ (originally from Genaissance now taken over by Cogenics) is the first commercially available, comprehensive genetic test for a heart rhythm disorder. This DNA test for cardiac ion channel mutations may remove uncertainty for the patients, their families, and their physicians with respect to establishing a diagnosis and can guide the physician in determining the best treatment options for those who are genetically predisposed to potentially fatal cardiac arrhythmias caused by long QT syndrome and related cardiac ion channel diseases. The test examines five cardiac ion channel genes for a mutation that is likely to cause long QT syndrome. If a genetic mutation is detected, its type and location can assist the physician in making treatment selections that could include lifestyle modification, prescription or avoidance of specific classes of drugs or the implantation of a defibrillator. A patient's family members also benefit from the test because it can identify if they inherited the same mutation as the initially symptomatic patient and may be at risk of a potentially fatal arrhythmia. These relatives often have ambiguous findings on an ECG, while the results of the FAMILION Test can answer whether or not they carry the familial mutation.

## *Gene Variant as a Risk Factor for Sudden Cardiac Death*

Extremes of the electrocardiographic QT interval, a measure of cardiac repolarization, are associated with increased cardiovascular mortality. A gene called NOS1AP (CAPON), which may predispose some people to abnormal heart rhythms leading to sudden cardiac death, was identified through a genome-wide association study (Arking et al. 2006). Statistically significant findings were validated in two independent samples of 2,646 subjects from Germany and 1,805 subjects from the USA Framingham Heart Study. NOS1AP, a regulator of neuronal nitric oxide synthase (nNOS), modulates cardiac repolarization. The gene, not previously discovered by traditional gene-hunting approaches, appears to influence significantly QT interval length as risk factor for sudden cardiac death. QT interval can be measured noninvasively with an EKG, and each person's QT interval, in the absence of a major cardiovascular event, is stable over time, making it a reliable measure. Approximately,

**Table 10.1** Genes that cause cardiovascular diseases

| Category | Disease | Gene | Function |
|---|---|---|---|
| Congenital malformations | Atrial septal defect | NKX2-5 | Transcription factor |
| | Holt–Oram syndrome (holes between the atria) | TBX5 | Transcription factor |
| Cardiomyopathy | Familial hypertrophic cardiomyopathy | βmyosin<br>Troponin T<br>Troponin I<br>Cardiac myosin binding protein C<br>α tropomyosin | Muscle contraction (forced generation) |
| | Idiopathic dilated cardiomyopathy | Actin<br>Dystrophin | Muscle contraction (force transduction) |
| Cardiac arrhythmias | Long QT syndrome | KLVQT1<br>HERG<br>minK | Potassium channel |
| | Idiopathic ventricular fibrillation (Brugada syndrome) | SCN5A | Sodium channel |
| | QT-related cardiac arrhythmia with sudden death | NOS1AP | Gene is regulator of neuronal nitric oxide synthase, which modulates cardiac repolarization |
| Myocardial infarction | Early onset | VAMP8 | Platelet degranulation |
| | Early onset | HNRPUL1 | Encodes a ribonuclear protein |
| Heart failure | Congestive heart failure | KIF6 wild-type gene | Kinesin family member 6 |
| Hypertension | Essential hypertension | AGT | Contraction of arterial smooth muscle |
| Blood lipid disorders | Familial hypercholesterolemia | LDL | Regulation of low-density lipoprotein |
| | Familial dyslipoproteinemias | ApoE | Regulation of plasma lipid concentrations |
| Atherosclerosis | Coronary artery disease | E-S128R | Monitors white blood cell adhesion to the arterial wall |
| | Coronary artery inflammatory disease | Interleukin-1 receptor antagonist (IL-1ra) gene | IL-1ra is a potent natural mechanism for controlling IL-1, and inflammation |
| Thrombotic disorders | Venous thrombosis<br>Stroke | Factor V (Leiden mutation) | Procoagulant normally by activated protein C |

60% of subjects of European ancestry carry at least one minor allele of the NOS1AP genetic variant, which explains up to 1.5% of QT interval variation.

Instead of focusing on so-called candidate genes with known functions that are highly suspect in heart beat rhythm, the researchers first focused on people who have extremely long or short QT intervals. They used subjects from two population-based studies, about 1,800 American adults of European ancestry from the Framingham Heart Study of Framingham, Massachusetts, and about 6,700 German adults from the KORA-gene study of Augsburg, Germany. They looked at SNPs that track with having a long or short QT interval. Only one particular SNP correlated with QT interval. That SNP was found near the NOS1AP gene, which has been studied for its function in nerve cells and was not previously suspected to play a role in heart function.

Identifying those at high risk for sudden cardiac death before fatalities occur has been challenging, both at the clinical and at the genetic level. In more than one third of all cases, sudden cardiac death is the first hint of heart disease. It is widely believed that many factors, genetic and environmental, contribute to irregular heartbeat and other conditions that may lead to sudden cardiac death. Now that variants of the NOS1AP gene have been correlated with QT interval length, the next project would be to figure out exactly how the DNA sequence variations alter the function of the gene, and how changes in gene function affects heart rhythm. Being able to identify predisposed individuals can help in saving their lives by prescribing beta-blockers and other drugs that regulate heart rhythm, and even by implanting automatic defibrillators in those with the highest risk.

## *KIF6 Gene Test as a Guide to Management of Congestive Heart Failure*

Carriers of the KIF6 (kinesin family member 6) wild-type gene are 50–55% more likely to develop CHF. KIF6 as a biomarker of CHF is the basis of a genetic test, StatinCheck, developed by Celera and offered through Berkeley HeartLab, which is owned by Celera. It is now licensed by clinical laboratory of Aurora Health Care in Milwaukee, Wisconsin.

Physicians can use the KIF6 test to identify the increased risk for CHF and begin treating their patients with statins. A study investigated whether 35 genetic polymorphisms, previously found to be associated with cardiovascular disease, were associated with MI in the CARE (Cholesterol and Recurrent Events) trial and with coronary heart disease (CHD) in the WOSCOPS (West of Scotland Coronary Prevention Study). In both the CARE and the WOSCOPS trials, carriers of the KIF6 719Arg allele had an increased risk of coronary events, and pravastatin treatment substantially reduced that risk (Iakoubova et al. 2008). Carriers of the 719Arg allele of KIF6 have 34% higher risk of MI and 24% higher risk of CHD compared with noncarriers among 25,283 women from the Women's Health Study, confirming and extending previous reports (Shiffman et al. 2008).

### *SNP Chip for Study of Cardiovascular Diseases*

Illumina is developing a custom SNP biochip for the study of vascular diseases through a collaboration with the Institute of Translational Medicine and Therapeutics (ITMAT) at the University of Pennsylvania, the Broad Institute at MIT, and the National Heart, Lung, and Blood Institute's (NHLBI) Candidate-gene Association Resource (CARe) Consortium. The IBC chip, named for ITMAT, Broad, and CARe, will be used to analyze more than 55,000 SNPs in genes that have been selected for cardiovascular-related phenotypes. The collaborators will use the Illumina iSelect Custom Genotyping BeadChip to study the genetic diversity of pathways for around 2,100 genes that are linked to vascular conditions including hypertension, myocardial infarction, heart failure, stroke, insulin resistance, metabolic disorders, dyslipidemia, and inflammation. The iSelect BeadChip enables scientists to train their research on specific SNPS-related to pathways or disease. The study plans to analyze more than 120,000 samples from population studies and clinical trials for possible links to vascular disease. The microarray will enable researchers to quickly genotype thousands of patients across thousands of genes to identify genetic risk factors underlying vascular diseases and other complex genetic traits.

## Pharmacogenomics of Cardiovascular Disorders

Application of pharmacogenomics for development of personalized treatment of cardiovascular disorders is illustrated by a few examples, such as myocardial infarction, heart failure, and hypertension, which are common conditions. The application of pharmacogenetics to cardiovascular disease management is also discussed. Factors that may be taken into account when selecting drug therapy for a patient with cardiovascular disease include age, race, concomitant diseases, medications, and renal and hepatic function. The renin-angiotensin system (RAS) plays a major role in the development and progression of cardiovascular diseases by promoting vasoconstriction, sodium reabsorption, cardiac remodeling, norepinephrine release, and other potentially detrimental effects. Angiotensin-converting-enzyme (ACE) inhibitors and angiotensin II type 1-receptor (AT1R) blockers are recommended for managing cardiovascular diseases, such as hypertension, myocardial ischemia, and heart failure. However, there is substantial variability in individual responses to these agents.

## Modifying the Genetic Risk for Myocardial Infarction

Variants in the 5-lipoxygenase–activating protein (FLAP) gene are associated with risk of myocardial infarction (MI). A randomized, prospective, placebo-controlled, crossover trial of DG-031 (DeCode Genetics Inc), an inhibitor of FLAP, was

conducted in MI patients who carry at-risk variants in the FLAP gene or in the leukotriene A4 hydrolase gene (Hakonarson et al. 2005). In patients with specific at-risk variants of two genes in the leukotriene pathway, DG-031 led to significant and dose-dependent suppression of biomarkers that are associated with increased risk of MI events. The investigators, however, do not know whether the drug's ability to suppress the biomarkers of inflammation would translate into a decreased risk of heart attack. There are some uncertainties about the rationale for the drug. One is that although some cardiologists theorize that inflammation is indeed a contributory cause of heart attacks, others regard it as just a symptom. If it is a symptom, a drug that reduced inflammation would do nothing to prevent heart attacks. Further research is needed to confirm the link between the gene variant and heart disease. If the drug proves effective, it could be taken as widely as the statin drugs. The average risk for a man older than 40 of having a heart attack at some time in his life is 49% and although just 33% percent of Americans have the at-risk variant, many more might gain a protective effect from the drug.

## Management of Heart Failure

### *β-Blockers*

β-blockers are recommended in addition to ACE inhibitors for the management of heart failure. A response to β-blockers therapy in heart failure has been associated with the ACE genotype. It appears that increased angiotensin II concentrations associated with the D allele may cause increased activation of the sympathetic nervous system and that patients with the D allele may thus derive greater benefits from pharmacologic interventions to decrease sympathetic nervous system activity (e.g., β-blocker therapy).

Despite the proven efficacy of β-blockers, there are many reasons why so many patients with congestive heart failure are not treated with these medications. One important reason is concern for adverse reactions, which occur in 25–43% of patients. Discontinuation of therapy is frequent due to hypotension, bradycardia, and worsening of heart failure. This has led to the study of genetic variants that determine response to β-blockers. Polymorphisms in the gene coding for the CYP2D6 isoenzyme, which catalyzes the metabolism of β-blockers such as metoprolol, carvedilol, timolol, and propranolol, may also affect β-blocker response. It is possible that the CYP2D6-related genotype interacts with drug target polymorphisms (e.g., β-receptor polymorphisms) and polymorphisms in genes involved in pathophysiology (e.g., the ACE I/D polymorphism) to influence the overall response to β-blockers.

In addition to genetic variants that affect plasma concentrations of a drug, variants in drug target, the $\beta_1$-receptor could also alter responses to β-blockers. A clinical study of titration of metoprolol controlled release/extended release in heart failure revealed that patients with the Gly389 variant and Ser49Ser genotype of $\beta_1$-receptor are significantly more likely to require increases in heart failure medications during

β-blocker titration and thus may require more frequent follow-up during titration (Terra et al. 2005).

## *Bucindolol*

Bucindolol's unique pharmacodynamics in advanced heart failure patients produce either a hyper-response (a beta1 receptor polymorphism) or avoid an adverse effect (an alpha2c receptor polymorphism). These dual gene loci create a set of diplotypes characterizing the population. By identifying important genetic factors underlying heart failure and the response to bucindolol, Arca Discovery Inc has identified those patients who will benefit most from bucindolol treatment. A polymorphism within a conserved beta(1)-adrenergic receptor motif alters cardiac function and β-blocker response in human heart failure. A study concluded that beta(1)AR-389 variation alters signaling in multiple models and affects the β-blocker therapeutic response in heart failure and, thus, might be used to individualize treatment of the syndrome (Liggett et al. 2006).

When prescribed genetically, bucindolol will be the state of the art in heart failure treatment for a majority of the of the US heart failure population. Bucindolol's unique pharmacology gives it other advantages as well, such as superior myocardial infarction clinical endpoints and tolerability.

## *BiDil*

Enalapril therapy is associated with a significant reduction in the risk of hospitalization for heart failure among white patients with left ventricular dysfunction, but not among similar black patients. This finding underscores the need for additional research on the efficacy of therapies for heart failure in black patients. This analysis, combined with other recent data from clinical trials, suggests that the overall population of black patients with heart failure may be underserved by current therapeutic recommendations. The fact that large-scale trials of therapy for heart failure have been performed in preponderantly white populations has limited the ability of the medical community to assess the efficacy of current therapies in black patients.

The relatively high level of heart failure in the black population has been attributed, in part, to a lack of nitric oxide (NO). BiDil (NitroMed), made of isosorbide dinitrate and hydralazine, is thought to reduce mortality in this population by restoring depleted NO levels, and by protecting NO that is formed naturally in vascular endothelial cells. A randomized trial has examined whether a fixed dose of Bidil provides additional benefit in blacks with advanced heart failure, a subgroup previously noted to have a favorable response to this therapy (Taylor et al. 2004). Hydralazine is an antioxidant and vasodilator, which means that it protects NO formed by isosorbide dinitrate and dilates blood vessels. Neither drug is indicated

separately for heart failure. The addition of a fixed dose of isosorbide dinitrate plus hydralazine to standard therapy for heart failure including neurohormonal blockers was shown to be efficacious and increased survival among black patients with advanced heart failure. The study was terminated early owing to a significantly higher mortality rate in the placebo group than in the group treated with the drug combination. NitroMed Inc has submitted the African American Heart Failure Trial (A-HeFT) clinical dataset to the FDA. The product was approved by the FDA in 2005. BiDil became the first drug to be developed and marketed on the basis of a demonstrated efficacy in black subjects and could pave the way for a generation of individualized medicines.

## Management of Hypertension

Hypertension is a common disorder affecting approximately 20% of the US population. Care of hypertensive patients vary a lot. Ideally, individual risks must be assessed in order for the best decision to be made as to which patients with hypertension to treat and how. Assessment identifies important cardiovascular risk factors that may warrant treatment and helps to establish the absolute benefits that patients can expect from particular treatments. The benefits of treating hypertensive patients also vary, depending on each patient's competing risks of dying from other than cardiovascular causes. For example, patients with multiple serious conditions, such as end stage Alzheimer's disease, obstructive lung disease, frequent falls, gout, and urinary incontinence, have high competing risks that may minimize or negate the benefits of treating their hypertension. Once the decision to treat has been made, an appropriate therapy should be selected.

Approximately, 100 medications are available for treatment in several categories: diuretics, α-blockers, β-blockers, aldosterone antagonists, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor antagonists, CNS active agents, and calcium channel blockers. Each of these categories contains several distinct drugs, which vary in their efficacy and liability to produce adverse reactions in different patient populations. β-adrenergic antagonists are generally recommended as first-line therapy, along with thiazide diuretics, for the treatment of hypertension. However, as many as 60% of hypertensive patients do not achieve adequate blood pressure lowering from monotherapy with β-blockers. It is plausible that genetic variation in the β-adrenergic-receptor genes accounts for some of the observed variability in blood pressure response.

### *Pharmacogenomics of Diuretic Drugs*

Diuretics are considered to be the first-line drugs for hypertension but their overall efficacy is not sufficient. Many patients suffer adverse effects such as disturbances

of serum K+levels. Variations in efficacy and susceptibility to adverse reactions of diuretics may be partially caused by genetic polymorphisms of genes involved in the pharmacodynamics and pharmacokinetics of diuretics. Genes with a role in the pharmacokinetics of most diuretics are renal drug transporters, especially OAT1, OAT3, and OCT2 (genes SLC22A6, SLC22A8, and SLC22A2) whereas variants in carbonic anhydrase (CA), cytochrome P450 enzymes, and sulfotransferases are relevant only for specific substances. Genes on the pharmacodynamic side include the primary targets of thiazide, loop, $K^+$-sparing, and aldosterone antagonistic diuretics: NCC, NKCC2, ENaC, and the mineralocorticoid receptor (genes SLC12A3, SLC12A1, SCNN1A, B, G, and NR3C2). Polymorphisms in these and in associated proteins such as GNB3, α-adducin and ACE seem to be clinically relevant.

A particular genetic alteration in hypertensive patients dramatically increases the risk of heart attack, stroke, or death, and may explain why some hypertensive patients fare worse than others, even if they take the same medication. Patients carrying α-adducin gene are less likely to suffer a heart attack or stroke if they were taking a diuretic. Data from the International Verapamil SR-Trandolapril study (INVEST-GENES) suggested that one genotype group benefited from the diuretic and had a reduction in heart attack and stroke, while the other genotype group did not. In the INVEST substudy, nearly a third of the participants were carriers of the tryptophan version of the alpha-adducin gene, a protein associated with the movement of ions, especially sodium, across cells. In these individuals, the amino acid glycine has been swapped with the amino acid tryptophan. Up to 40% of the population carries at least one copy of the tryptophan form of the gene. Patients with this version had a 43% higher risk of heart attack, stroke, or death than those with the glycine form in the 2½ years after the study began. But unlike previous research, the UF study did not show that patients with the glycine form benefited more from diuretics, which help lower blood pressure by removing excess salt and water from the body. The findings of this study may enable patients to receive appropriate personalized medicine based on their genetic makeup.

## *Pharmacogenomics of ACE Inhibitors*

Polymorphism of the ACE gene is known to influence the response to ACE inhibitor fosinopril in hypertensive patients. Blacks with hypertension, as a group, have lower plasma renin activity and are less likely than hypertensive whites to achieve adequate blood pressure reductions with ACE inhibitor monotherapy. Hypertension is considered to be a good model for development of personalized medicine because it is a multifactorial disease.

It is now possible to identify a subgroup of hypertensive patients (30%) that should be treated with ACE-inhibitors as first line of treatment, since they will show a much better response than the remaining population. This test has been expanded to cover a panel of different classes of antihypertensive treatments, such as angiotensin II antagonists and β-blockers. Such a test enables the selection of the

most efficacious drug as first line of treatment leading to reduction of the number of drugs required for adequate treatment as well the number of visits by the patient to the health care facility for monitoring of blood pressure. The overall effect would be improvements in quality of health care and cost savings.

## *Management of Hypertension by Personalized Approach*

Despite the many therapeutic options for hypertension, only 27% of the patients achieve adequate control of blood pressure. Therefore, there is an opportunity to improve the management of hypertension through pharmacogenomics-based personalized medicines as shown in Fig. 10.1.

Being a polygenic disorder, hypertension still remains a challenge for designing better future treatments. Largest and most recent searches of the genome have found limited evidence of genes that determine hypertension. Linkage analysis identified a principle locus on chromosome 6q, with a lod score of 3.21 that attained genome-wide significance (Caulfield et al. 2008). The discovery of a single allele proven to be associated with control of blood pressure could lead to the discovery of relevant and novel targets for prevention and treatment of hypertension (Harrap 2003).

Pharmacogenetic-guided therapy has clinical potential for management of hypertension, but there are few controlled studies on this topic. A clinical trial on individuals with uncomplicated hypertension aims to identify the genetic determinants of the antihypertensive and adverse metabolic responses to a thiazide diuretic (hydrochlorothiazide), a beta-blocker (atenolol), and their combination (Johnson et al. 2009). This will be accomplished through candidate gene and genome-wide

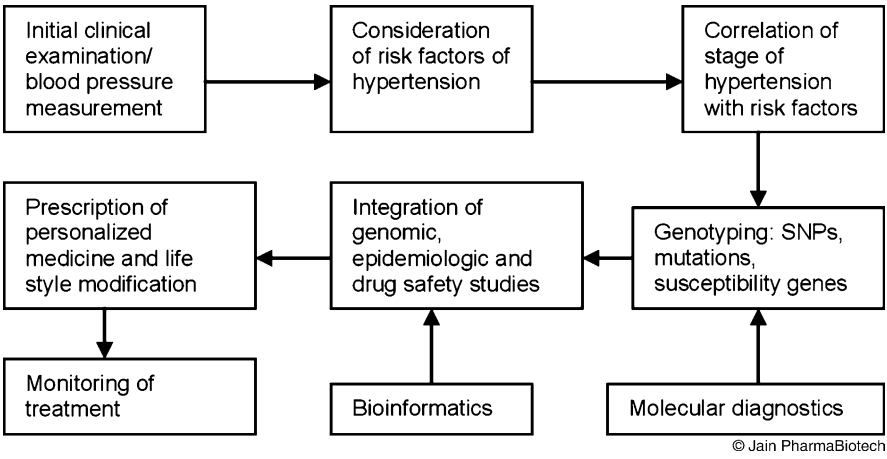


**Fig. 10.1** A scheme of personalized approach to management of hypertension

association approaches. Current antihypertensive therapy is discontinued, and hypertension is confirmed, along with collection of other baseline data. Subjects are then randomized to either hydrochlorothiazide or atenolol, with one dose titration step, followed by assessment of response to therapy after at least 6 weeks on the target dose. Those with blood pressure >120/70 mmHg have the second drug added, with similar dose titration and response assessment procedures. Data collected include home, office, and 24 h ambulatory blood pressure. Biological samples collected in the fasting state include plasma, serum, DNA, and urine. This trial will add substantially to our understanding of the genetic determinants of antihypertensive and adverse metabolic responses to two commonly used antihypertensive drug classes.

## *Prediction of Antihypertensive Activity of Rostafuroxin*

Two mechanisms, among others, are associated with essential hypertension and related organ damage: mutant α-adducin variants and high concentrations of endogenous ouabain. An antihypertensive agent, rostafuroxin, selectively inhibits these mechanisms in rodents. A study has investigated the molecular and functional effects of mutant α-adducin, ouabain, and rostafuroxin in hypertensive rats, human cells, and cell-free systems and demonstrated that both mutant α-adducin variants and the ouabain–Na,K-ATPase (Na+− and K+−dependent adenosine triphosphatase) complex can interact with the Src-SH2 (Src homology 2) domain, increasing Src activity and the Src-dependent Na,K-ATPase phosphorylation and activity (Ferrandi et al. 2010). Wild-type α-adducin or Na,K-ATPase in the absence of ouabain showed no interaction with the Src-SH2 domain. Rostafuroxin disrupted the interactions between the Src-SH2 domain and mutant α-adducin or the ouabain–Na,K-ATPase complex and blunted Src activation and Na,K-ATPase phosphorylation, resulting in blood pressure normalization in the hypertensive rats.

The translatability of these data to humans was also shown in a pharmacogenomic clinical trial, which investigated the relationship between variants of genes encoding enzymes for ouabain synthesis (lanosterol synthase) and HSD3B1 (hydroxy-δ-5-steroid dehydrogenase, 3β- and steroid δ-isomerase 1), ouabain transport and adducin activity, and the responses to antihypertensive medications. (Lanzani et al. 2010). The genetic profile defined by these variants predicted the antihypertensive effect of rostafuroxin, a sodium pump blocker, but not that of losartan or hydrochlorothiazide. The magnitude of the rostafuroxin antihypertensive effect was twice that of antihypertensive drugs recently tested in phase II clinical trials. One quarter of patients with primary hypertension display these variants of adducin or concentrations of endogenous ouabain and would be expected to respond to therapy with rostafuroxin. Because the mechanisms that are inhibited by rostafuroxin also underlie hypertension-related organ damage, this drug may also reduce the cardiovascular risk in these patients beyond that expected by the reduction

in systolic blood pressure alone. The results open up the possibility of improved patient stratification, as they allow predictions to be made about the effectiveness of rostafuroxin (but not that of any other antihypertensive drugs) in patients carrying key gene variants.

## Pharmacogenetics of Lipid-Lowering Therapies

Cardiovascular disease is associated with nonmodifiable risk factors such as age, gender, and genetic background, and with modifiable risk factors such as lipid concentrations. Lowering serum lipid levels has been demonstrated to slow the progression of, or even induce regression in, atherosclerosis. However, like any other drug treatment, the magnitude of plasma lipid responses to drug therapies varies considerably among individuals modified by a number of factors such as age, gender, concomitant disease, and genetic determination. Pharmacogenetics provides the experimental basis to understand the variability in response to drugs as a function of the individual genetic makeup. Information from small clinical trials reveals that several candidate genes may hold some promise in our quest to predict individual success to hypolipemic drug treatment.

### *Polymorphisms in Genes Involved in Cholesterol Metabolism*

Polymorphisms in genes involved in cholesterol synthesis, absorption, and transport may affect statin efficacy. Genetic variation at the LDL receptor locus can affect baseline lipids, response to pravastatin, and cardiovascular disease risk in subjects placed on statin treatment (Polisecki et al. 2008). The DNA of 1,536 individuals treated with pravastatin, was analyzed for 148 SNPs within ten candidate genes related to lipid metabolism (Chasman et al. 2004). Variation within these genes was then examined for associations with changes in lipid levels observed with pravastatin therapy. Two common and tightly linked SNPs were significantly associated with reduced efficacy of pravastatin therapy. Both of these SNPs were in the gene coding for 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the target enzyme that is inhibited by pravastatin. The association for total cholesterol reduction persisted even after adjusting for multiple tests on all 33 SNPs evaluated in the HMG-CoA reductase gene as well as for all 148 SNPs evaluated was similar in magnitude and direction among men and women and was present in the ethnically diverse total cohort as well as in the majority subgroup of white participants. Thus, individuals heterozygous for a genetic variant in the HMG-CoA reductase gene may experience significantly smaller reductions in cholesterol when treated with pravastatin. The absolute difference in total cholesterol reduction associated with HMG-CoA reductase was significant enough to affect

health outcome. Future studies should determine if this difference can be offset by adjustment of dose or use of a non-statin cholesterol-lowering agent.

There is interindividual variation in low-density lipoprotein cholesterol (LDLc) lowering by statins. An intronic SNP in ABCA1 and the APOE ε3 allele are associated with reduced LDLc lowering by statins and identify individuals who may be resistant to maximal LDLc lowering by statins (Voora et al. 2008).

HMG-CoA reductase inhibitors are generally very well tolerated but there are two uncommon but potentially serious adverse effects related to HMG-CoA reductase inhibitor therapy: hepatotoxicity and myopathy. The occurrence of lethal rhabdomyolysis in patients treated with cerivastatin has prompted concern on the part of physicians and patients regarding the tolerability of HMG-CoA reductase inhibitors. CYP2D6 plays an important role in the metabolism of simvastatin. It has been shown that the cholesterol-lowering effect as well as the efficacy and tolerability of simvastatin are influenced by CYP2D6 genetic polymorphism. Because the different HMG-CoA reductase inhibitors differ with respect to the degree of metabolism by the different CYP enzymes, genotyping may help to select the appropriate HMG-CoA reductase inhibitor and the optimal dosage during the start of the treatment and will allow for more efficient individual therapy (Vermes and Vermes 2004).

## *Role of eNOS Gene Polymorphisms*

The eNOS gene harbors a common polymorphism in intron 4 (4a/b), and some clinical studies have suggested an association of the rare a-allele with coronary artery disease and myocardial infarction. However, contradictory results have also been reported. One study has investigated the associations of eNOS polymorphism with these diseases in two prospective autopsy series comprising altogether 700 Caucasian Finnish men who died suddenly (Kunnas et al. 2002a). In ANCOVA, no significant differences in areas of atherosclerotic lesions and coronary stenosis percentages were found between men carrying the a-allele (ba + aa) compared with those homozygous for the b-allele. Subjects with the a-allele had significantly lower risk of myocardial infarction compared with those carrying the bb genotype. Men with the a-allele also tended to have coronary thrombosis less often. The eNOS gene 4a/b polymorphism was not associated with the extent of coronary atherosclerosis, but the a-allele of the variant seems to protect to some degree against the development of myocardial infarction. In a placebo-controlled study, adenosine-stimulated myocardial perfusion, as determined by PET, improves after treatment with pravastatin in subjects with the eNOS ba-genotype but not in those with the bb-genotype (Kunnas et al. 2002b). This effect is not dependent on the decrease of serum cholesterol.

However, the current clinical relevance of this knowledge is quite limited due to the small effects observed for each of the genetic markers examined. Future progress in this area will be driven by studying gene–gene and gene–treatment interactions in much larger patient populations (Ordovas and Shen 2002).

### *The STRENGTH Study*

The STRENGTH study (Statin Response Examined by Genetic HAP Markers) was launched in 2001 by Genaissance Inc (acquired by Clinical Data Inc). It is the largest prospective clinical trial ever conducted to discover how physicians can personalize prescriptions using information about human genomic variation. As the earliest application of pharmacogenomics to one of the most prevalent public health problems – hypercholesterolemia – the study is designed to provide the information necessary for physicians to decide which cholesterol lowering drug is best for each patient based on their own genetic makeup. The four drugs under study were: Lipitor (atorvastatin), Zocor (simvastatin), Pravachol (pravastatin), and Baycol (cerivastatin). In 2002, new results were announced from ongoing analyses of the STRENGTH I clinical study that further demonstrate the ability of its HAP Technology to identify specific genetic markers (gene haplotypes or HAP Markers) that are associated with the effects of statin therapy, including LDL (bad) cholesterol, HDL (good) cholesterol, and triglycerides.

The study has examined 100 genes so far from three functional pathways (lipid metabolism and transport, inflammation, and drug metabolism). A total of 29 HAP Markers from 27 of these genes were found to have statistically significant associations with clinical response (LDL, HDL, and/or triglycerides) to simvastatin, atorvastatin, or pravastatin. Each HAP Marker occurred in at least 10% of the study group and, thus, may have wide clinical applicability across the population of patients with hyperlipidemia. Twenty-five of the markers were linked to outcomes for specific drugs and four were associated with the effects of statins as a drug class. These important findings highlight the differences between drugs in the statin class and clearly indicate the need and the potential to optimize therapy based on the genetics of different patient populations. The medical community has been aware of clinical and metabolic differences among the statins but this study provided some genetic evidence that begins to explain these differential effects.

Two examples of drug-specific markers are based on a deeper analysis that was completed for 25 genes. A marker from one gene was associated with a positive HDL cholesterol clinical response in one of the statins, a negative response in a second drug and no change in HDL response in a third. It is notable that the same marker predicts opposing effects for two of the drugs. This marker, found in 26% of the STRENGTH patients, illustrates how differently the drugs interact with biological pathways and may help to explain the variation in the HDL response of patients when taking different statins. In this study, this one predictive marker is as powerful as all of the other currently available predictors put together, including age, alcohol use, smoking, gender, body mass index, exercise, and baseline HDL levels. Further analyses of STRENGTH data include the evaluation of additional markers for drug efficacy as well as markers that may be predictive of side effects, such as muscle damage. Further clinical studies have continued on this topic (Jain 2011).

Marked lowering of low-density-lipoprotein cholesterol (LDL-C) levels (< or = 50%) with intensive statin therapy is associated with major reduction in cardiovascular risk, but is limited by a potential increase in adverse effects, thereby justifying

optimization of LDL-C reduction with minimal risk. The organic anion transporting polypeptide-1B1 encoded by the SLCO1B1 gene is implicated as a major transporter in cellular uptake of statins, and notably fluvastatin. Results of a pharmacogenomics study on elderly subjects with hypercholesterolemia reveal that OATP1B1 gene is implicated in the pharmacological action and efficacy of fluvastatin (Couvert et al. 2008). The common *14 allele of SLCO1B1, which is distinguished by the presence of the c.463 C>A polymorphism, was associated with enhanced lipid-lowering efficacy in this study.

### *Personalized Management of Women with Hyperlipidemia*

A study conducted by Genaissance Pharmaceuticals (taken over by Clinical Data) on individuals who were candidates for statin therapy suggests that women with a genetic predisposition to protective levels of C-reactive protein (CRP, an established marker for fatal coronary disease) lose that benefit when taking hormone replacement therapy (Judson et al. 2004). The results of this study are the first published result from Genaissance's STRENGTH study described in the preceding section.

Several studies by leading academic/medical centers have shown that CRP levels may be more important than cholesterol levels for predicting cardiovascular events such as heart attacks. In particular, these studies have shown that elevated CRP is a risk factor that is independent of cholesterol levels. It had previously been shown that HRT caused elevated levels of CRP and heart attacks and strokes (Women's Health Initiative). The current study shows that the protective effect of a key genetic variant may be overwhelmed by the use of these drugs.

The results give lifestyle guidance to women who would like to preserve the protective benefits conferred by favorable genetic variations, and may ultimately lead to new or modified drugs. The study showed that men and women with common variants in the apolipoprotein E (APOE) gene on average have naturally lower levels of CRP. In the case of women, however, the study indicates that this beneficial effect may be largely neutralized by HRT, allowing CRP levels to potentially increase to dangerous levels.

## Thrombotic Disorders

A number of thrombotic disorders cause cardiovascular disease. Venous thrombosis has an annual incidence of 1 per 1,000 in the general population and is associated with significant morbidity and mortality. Several genetic variants have been identified that are associated with an increased risk of venous thrombosis, including a recently discovered mutation in the prothrombin gene. Factor V Leiden mutation is associated with 15–20% of the cases of idiopathic thrombotic disorders.

## *Factor V Leiden Mutation*

A mutation in the procoagulant protein Factor V (Factor V Leiden) causes it to be relatively resistant to degradation by activated protein C (APC), resulting in a thrombotic tendency. The mutation is a guanine-to-adenine substitution at nucleotide 1,651 that results in a glutamine-to-arginine substitution at position 506 (R506Q). This is a clinically significant mutation, since it is relatively common (found in 3–6% of Caucasian subjects) and has been shown to be associated with venous thrombosis and stroke. It is of special importance in women for the following reasons:

- It increases the risk of venous thrombosis associated with oral contraceptives and hormone replacement therapy.
- It synergizes with pregnancy, which by itself increases the risk of venous thrombosis.
- It is associated with intrauterine growth restriction, still births, and cerebral palsy in the off-spring.
- It is associated with myocardial infarction in young women but not in young men.

This mutation can be readily detected by molecular diagnostics. The presence of Factor V mutation is an important consideration for anticoagulant therapy to prevent thromboembolism and should be individualized for each patient. CYP2C9 mutation is a predicator for anticoagulation-related therapy in these patients.

## *Anticoagulant Therapy*

Warfarin is widely used to prevent thromboembolic events in patients with atrial fibrillation, prosthetic heart valves, and previous cerebrovascular events. Warfarin is a narrow therapeutic index drug; inadequate or excessive anticoagulation may result in substantial morbidity and potentially death because of thromboembolic complications or bleeding. Warfarin therapy is complicated by great interpatient variability in the dosage needed to achieve optimal anticoagulation.

Several genes play a role in warfarin's metabolism. The S-isomer of warfarin has five times the anticoagulant activity of the R-isomer and is metabolized by CYP2C9. Polymorphisms in CYP2C9, a gene for cytochrome P450, cause about 30% of patients to be slow warfarin metabolizers, which could result in high blood concentrations (Gage 2004). In a study of orthopedic patients, Gage showed that testing for CYP2C9 polymorphisms does provide a better starting point for the warfarin dose, which would achieve stable blood levels more quickly than trial-and-error dosing. Many Caucasians (~50%) possess less active forms of CYP2C9, a key enzyme in warfarin metabolism: tenfold interpatient variability in the dose of warfarin required to attain a therapeutic response. Frequent assessment of anticoagulation status is necessary during warfarin therapy to ensure drug efficacy and to prevent or minimize hemorrhagic events. Thus, the identification of factors that

influence warfarin dosage requirements would be of great benefit in the management of patients at risk for coagulation disorders. Polymorphisms in the vitamin K epoxide reductase multiprotein complex (VKOR) also affect warfarin metabolism in rats (Rost et al. 2004). Mutations in one of the complex's subunits, VKORC1, confer warfarin resistance in some human disorders. Overexpression of the wild-type protein made rats sensitive to the treatment. Future studies will genotype for both CYP2C9 and VKORC1 when prescribing warfarin before surgery.

Heparin is used to prevent and treat thromboembolic diseases. One of the most serious adverse reactions to heparin is an immune-related thrombocytopenia. Heparin-induced thrombocytopenia (HIT) can result in severe thromboembolic complications and death. Heparin-induced antibodies recognize and bind to heparin-platelet factor 4 complexes and subsequently activate platelets via the platelet Fc-receptor to mediate HIT. A single-nucleotide polymorphism commonly occurs in the platelet Fc-receptor gene, resulting in an arginine or histidine at codon 131 (131Arg/His), and appears to affect platelet aggregation.

### *Antiplatelet Therapy*

Prasugrel, an approved antiplatelet drug for cardiovascular thrombotic disease, is a prodrug with rapid and almost complete absorption after oral ingestion of a loading dose. It is metabolized into its active form, which binds irreversibly to the adenosine diphosphate (ADP) P2Y12 receptor on platelets for their lifespan, thereby inhibiting their activation and decreasing subsequent platelet aggregation. Hydrolysis by intestinal carboxylesterases and oxidation by intestinal and hepatic cytochrome P-450 enzymes convert prasugrel into its active metabolite. Prasugrel has a greater antiplatelet effect than clopidogrel because it is metabolized more efficiently. Genetic polymorphisms affecting the cytochrome P450 system may explain some of the differences in metabolism between prasugrel and clopidogrel.

## Nanotechnology-Based Personalized Therapy of Cardiovascular Diseases

The future of cardiovascular diagnosis is already being impacted by nanosystems that can both diagnose pathology and treat it with targeted delivery systems (Wickline et al. 2006). The potential dual use of nanoparticles for both imaging and site-targeted delivery of therapeutic agents to cardiovascular disease offers great promise for individualizing therapeutics. Image-based therapeutics with site-selective agents should enable verification that the drug is reaching the intended target and a molecular effect is occurring. Experimental studies have shown that binding of paclitaxel to smooth muscle cells in culture has no effect in altering the growth characteristics of the cells. If paclitaxel-loaded nanoparticles are applied to the cells, however, specific binding elicits a substantial reduction in smooth muscle cell proliferation, indicating that

selective targeting may be a requirement for effective drug delivery in this situation. Similar behavior has been demonstrated for doxorubicin containing particles. Intravenous delivery of fumagillin (an antiangiogenic agent)-loaded nanoparticles targeted to αvβ3-integrin epitopes on vasa vasorum in growing plaques results in marked inhibition of plaque angiogenesis in cholesterol-fed rabbits. The unique mechanism of drug delivery for highly lipophilic agents such as paclitaxel contained within emulsions depends on close apposition between the nanoparticle carrier and the targeted cell membrane and has been described as "contact facilitated drug delivery." In contrast to liposomal drug delivery (generally requiring endocytosis), the mechanism of drug transport in this case involves lipid exchange or lipid mixing between the emulsion vesicle and the targeted cell membrane, which depends on the extent and frequency of contact between two lipidic surfaces. The rate of lipid exchange and drug delivery can be greatly increased by the application of clinically safe levels of ultrasound energy that increase the propensity for fusion or enhanced contact between the nanoparticles and the targeted cell membrane.

The combination of targeted drug delivery and molecular imaging with MRI has the potential to enable serial characterization of the molecular epitope expression based on imaging readouts. Monitoring and confirmation of therapeutic efficacy of the therapeutic agents at the targeted site would permit personalized medical regimens.

## Project euHeart for Personalized Management of Heart Disease

In 2008, the European Union (EU) funded a research project called "euHeart," which is aimed at improving the diagnosis, therapy planning, and treatment of cardiovascular disease. euHeart project complements the earlier HeartCycle project, which focuses on the long-term management of chronic heart disease patients. The euHeart consortium, led by Philips Healthcare, aims to develop advanced computer models of the human heart that can be personalized to patient-specific conditions using clinical data from various sources, such as CT and MRI scans, measurements of blood flow and blood pressure in the coronary arteries, and ECGs. These computer models will integrate the behavior of the heart and the aorta at molecular, cellular, tissue, and organ-level. They will also incorporate clinical knowledge about how cardiovascular disease disturbs the correct functioning of the heart at these levels. As a result, it may be possible to develop simulation tools that physicians can use to predict the outcome of different types of therapy, and because the models will be personalized to individual patients, the therapy could be equally personalized.

As an example, one way of treating heart rhythm disorders is a minimally invasive procedure known as radio-frequency ablation. During this procedure, a catheter is inserted into the patient's heart and the tissue responsible for propagating abnormal electrical signals through the heart muscle is destroyed using heat from a radio-frequency field generated at the tip of the catheter. Currently, physicians have to rely on their experience to decide which areas of tissue to destroy – a task that is

complicated by the fact that the electrical activity in every patient's heart is subtly different. With the aid of a computerized model that reflects the patient's unique heart structure and function, it may be able to test the results of destroying different areas of tissue before operating on the patient.

## Concluding Remarks

Genetic factors may influence the response to antihypertensive medication. A number of studies have investigated genetic polymorphisms as determinants of cardiovascular response to antihypertensive drug therapy. Hypertensive patients with the 460 W allele of the α-adducin gene have a lower risk of myocardial infarction and stroke when treated with diuretics compared with other antihypertensive therapies. With regard to blood pressure response, interactions were also found between genetic polymorphisms for endothelial nitric oxide synthase (eNOS) and diuretics and the ACE gene and angiotensin II type 1 receptor antagonists. Although there are controversies to settle and difficulties to overcome, pharmacogenetics may yield successful strategies to optimize drug therapy. Several candidate genes are currently under investigation for their potential to modify response to antihypertensive drugs. Findings from previous studies require conformation in other studies to be able to make definitive conclusions about current positive drug–gene interactions. It is also important that research groups collaborate more in order to facilitate the conduct of a meta-analysis for conclusive results. With the development of efficient methods for analyzing massive amounts of data, pharmacogenetic studies may eventually lead to the optimization of antihypertensive drug therapy based on genetic profiles of patients.

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