Overview of recent advances in molecular cardiology

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Molecular cardiology is a new and fast-growing area of cardiovascular medicine that aims to apply molecular biology techniques for the mechanistic investigation, diagnosis, prevention and treatment of cardiovascular disease. As an emerging discipline, it has changed conceptual thinking of cardiovascular development, disease etiology and pathophysiology. Although molecular cardiology is still at a very early stage, it has opened a promising avenue for understanding and controlling cardiovascular disease. With the rapid development and application of molecular biology techniques, scientists and clinicians are closer to curing heart diseases that were thought to be incurable 20 years ago. There clearly is a need for a more thorough understanding of the molecular mechanisms of cardiovascular diseases to promote the advancement of stem cell therapy and gene therapy for heart diseases. The present paper briefly reviews the state-of-the-art techniques in the following areas of molecular cardiology: gene analysis in the diseased heart; transgenic techniques in cardiac research; gene transfer and gene therapy for cardiovascular disease; and stem cell therapy for cardiovascular disease.

Key Words: DNA; Genes; Heart diseases; Hypertrophy; Molecular biology; Myocardial infarction

Rapid advances in molecular biology in the past decade have changed the practice of clinical cardiology by increasing understanding of molecular mechanisms of cardiovascular disease, and providing new diagnostic and therapeutic tools. It is expected that knowledge in molecular cardiology will grow exponentially in the next few years. Already, molecular and genetic cardiology has increased awareness of the inheritance of defective genes and their impact on cardiovascular disease. Subsequently, there have been numerous attempts to apply this new knowledge at the bedside. However, routine molecular genetic testing or DNA analysis is not available in most hospitals or clinical chemistry laboratories. In addition, there are few powerful molecular biology tests and gene therapy protocols established for treating cardiovascular diseases, mainly due to a lack of dependable techniques. Thus, there is an urgent demand for the advancement of efficient and dependable molecular cardiology techniques.

The development of molecular cardiology depends on the availability of established molecular biology techniques. There have been several major advances in the area of molecular biology that have contributed significantly to the field of molecular cardiology: the discovery and application of specific restriction endonucleases and reverse transcriptase; the development of cloning techniques; the availability of rapid DNA

Un survol des récents progrès de la cardiologie moléculaire

La cardiologie moléculaire est un nouveau domaine en forte expansion de la médecine cardiovasculaire, qui vise à appliquer les techniques de biologie moléculaire aux explorations mécanistes, au diagnostic, à la prévention et au traitement des maladies cardiovasculaires. Cette discipline émergente a modifié la pensée conceptuelle du développement cardiovasculaire ainsi que de l'étiologie et de la physiopathologie des maladies. Bien que la cardiologie moléculaire soit encore à un stade très précoce, elle a ouvert des voies prometteuses pour comprendre et contrôler les maladies cardiovasculaires. Grâce au développement et à l'application rapides des techniques de biologie moléculaire, les scientifiques et les cliniciens se rapprochent de la guérison de maladies cardiaques qu'on croyait incurables il y a 20 ans. De toute évidence, il faut comprendre les mécanismes moléculaires des maladies cardiaques de manière plus approfondie pour promouvoir la progression de la thérapie des cellules souches et de la thérapie génique dans les maladies cardiaques. Le présent article analyse brièvement les techniques de pointe dans certains domaines de la cardiologie moléculaire : analyse génétique du cœur malade, techniques transgéniques en recherche cardiaque, transfert et thérapie géniques ainsi que thérapie des cellules souches en présence de maladies cardiovasculaires.

sequencing; the improvement in vector technology; and the completion or near completion of human and animal genomes. These advances will allow us to understand the gene regulation of cardiac and vascular growth, identify genes responsible for cardiovascular diseases, perform diagnostic in situ hybridization and develop gene therapy approaches to cardiovascular diseases.

The present review covers the following four areas: gene analysis in the injured and hypertrophied heart; transgenic techniques in cardiac research; gene transfer and gene therapy for cardiovascular disease; and stem cell therapy for cardiovascular disease.

GENE ANALYSIS IN THE DISEASED HEART

There is increasing evidence indicating that cardiovascular diseases are associated with changes in gene expression. Identification of new candidate genes involved in heart diseases will provide the molecular basis for diagnosis, prevention and intervention. It is important to determine if the changes in gene expression cause heart diseases or are secondary to heart disease.

Cardiac hypertrophy is the most common risk factor contributing to cardiovascular mortality and morbidity. The pathogenesis of cardiac hypertrophy is multifactorial, with

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genetic background and environmental stress as two critical components. In some cases, the development of cardiac hypertrophy is the result of compensatory responses of the heart to increased hemodynamic load. However, some forms of cardiac hypertrophy are independent of pressure overload (1,2). Endocrine factors (catecholamines, angiotensin II, thyroxine hormone, cytokines, etc) or primary genetic abnormalities can cause cardiac hypertrophy. It is possible that cardiac hypertrophy is a multigenic disease.

It should be mentioned that different mutations inducing hypertrophic cardiomyopathy are associated with different prognoses and survival times. With respect to the genetic diagnosis of cardiac diseases, such as hypertrophic cardiomyopathy or the long QT syndrome, it has become possible to characterize genetic mutations responsible for some heart diseases. Rajamanickam and Jeejabai (3,4) have described methods and protocols for delineating and evaluating genes involved in cardiac hypertrophy in response to pressure overload. Perhaps a better way to determine the role of a specific gene in pressure-overload hypertrophy is to evaluate the hypertrophic response of rodents lacking or overexpressing this gene (5). One study (2) found that angiotensinogen gene knockout did not affect the development of cardiac hypertrophy in cold-exposed mice, suggesting that the renin-angiotensin system is not involved in cold-induced cardiac hypertrophy, a form of hypertrophy independent of pressure overload (6). Similarly, mice lacking AT_{1A} receptors have been found to develop cardiac hypertrophy to the same extent as wild-type mice do in response to aortic banding or chronic cold exposure (7,8), indicating that AT_{1A} receptors do not play a role in these models of cardiac hypertrophy. However, the renin-angiotensin system and AT_{1A} receptors have been reported to play a role in some forms of cardiac hypertrophy (9,10). For example, upregulation of AT_1 receptor gene may be involved in human cardiac hypertrophy (11). Thus, molecular mechanisms mediating hypertrophic responses vary with models of cardiac hypertrophy.

Recent studies indicate that microarray analysis of gene expression is a useful tool for mapping gene expression profiles in the injured or hypertrophied heart. Yanagawa et al (12) provided technical information and experimental protocols for using oligonucleotide analysis of gene expression. It should be emphasized that several intracellular signalling pathways are known to be involved in the pathogenesis of cardiac hypertrophy (13). Methods for detecting cardiac signalling in the hypertrophied heart have been detailed by Si et al (13).

Knowledge of the role of apoptosis in heart diseases will help in the development of approaches for controlling apoptosis, which is believed to be involved in cardiovascular abnormalities such as cardiac hypertrophy, myocardial infarction (MI) and atherosclerosis. Hunter et al (14) have documented the widely used protocols for detecting and quantifying apoptosis in cardiovascular tissues. Altered beta-adrenergic receptor gene regulation and signalling can result in cardiac malfunctions. The beta-adrenergic receptor is a well known therapeutic target for cardiac dysfunction. Methods for the detection of altered beta-adrenergic signalling pathways in hypertrophied hearts have been recently described by Wolf et al (15). The protooncogene, *c-myc*, mediates cell growth and proliferation in many cell types. Inducible activation of *c-myc* in adult myocardium in vivo provokes cardiac myocyte hypertrophy and reactivation of DNA synthesis (16). Enhanced expression

of *c-myc*, *c-fos* and *h-ras* genes are found in cardiac hypertrophy and cardiac dilation in patients (17). Thus, biomechanical stress-induced cardiac myocyte hypertrophy is accompanied by changes in several intracellular signals, including oncogene expression.

It should be mentioned that changes in messenger RNA expression levels do not necessarily reflect changes in protein expression levels. Therefore, it is important to determine the functional protein expression. The recent application of proteomic techniques has greatly increased our understanding of the heart disease process. Two-dimensional polyacrylamide gel electrophoresis can separate thousands of proteins in a few steps (18). Mass spectrometry has been used for the identification of proteins and their post-translational modifications (19). In addition, functional proteomics provides structural and functional assessments of multiprotein complexes such as integrated chloride channel in the heart (20). Thus, cardiovascular proteomics has emerged as a new discipline that will have a great impact on the diagnosis and treatment of cardiovascular disease.

TRANSGENIC TECHNIQUES IN CARDIAC RESEARCH

In recent years, rapid advances in molecular genetics have heralded a new era of genetic cardiology to study cardiovascular function and disease at the molecular level. Since the first cardiac-specific transgenic mouse was created in 1988 (21), hundreds of transgenic or gene-targeted murine models have been generated for the overexpression, genetic ablation or site-specific mutation of key proteins governing cardiac structure and function. Transgenic techniques are powerful and valuable tools for cardiovascular research where transgenic animal models are produced to mimic human cardiovascular diseases. This has provided scientists with a unique approach to the study of the role of a particular gene in cardiovascular disease. However, transgenic animals are not available to all cardiovascular researchers and most laboratories are unable to generate particular transgenic animal models when needed due to technical difficulties. Thus, it is imperative to document updated techniques and easy-to-follow protocols for successful production of transgenic models for cardiac research.

Babu and Periasamy (22) have recently described technical protocols necessary for the generation of mouse models of cardiac dysfunction by overexpressing different sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) isoforms and a SERCA2 knockout mouse model with decreased SERCA levels (22). To study cardiomyocyte defects in diabetes, Shen et al (23) have updated the procedures for maintenance and breeding of two diabetic animal models, OVE26 and Agouti mice, for type I and type II diabetes, respectively. Protocols for producing cardiac-targeted transgenes have been described by Hoffman (24). The transgenic rat overexpressing the AT_1 receptor in cardiomyocytes offers an excellent paradigm for studying a target gene in a specific type of cell or tissue (24). It is expected that these exemplary methods will stimulate the creation of additional novel transgenic animal models for cardiac research. The generation of transgenic animals and the subsequent phenotypic characterization of these genetic manipulations in intact animals and isolated hearts have greatly enhanced our current knowledge of cardiac development, Ca^{2+} handling, excitation-contraction coupling, receptor-mediated signal transduction and the heart disease process.

Ultimately, these advances will help identify optimal therapeutic targets for heart diseases.

However, cautions should be given when performing functional analyses in animals with conventional global gene knockout or knockin because it may result in compensational functional changes. The ideal approach for analyzing the function of a specific gene in the heart would be to use heart-specific conditional or inducible gene knockout or knockin animal models. These models may be more valuable in the phenotypic study of a specific gene because conditional or inducible gene knockout or knockin limits the effect of up-regulation or developmental compensation associated with genetic manipulation. For example, to specifically assess the role of the mineralocorticoid receptor (MR) in the heart, Ouvrard-Pascaud et al (25) generated a transgenic mouse model with conditional cardiac-specific overexpression of human MR. Cardiac MR overexpression led to ion channel remodelling, prolonged ventricular repolarization at both the cellular and integrated levels, and severe ventricular arrhythmia. The results indicate that cardiac MR can trigger cardiac arrhythmias, suggesting that MR antagonism may be a novel approach for the treatment of arrhythmia.

GENE TRANSFER AND GENE THERAPY FOR CARDIOVASCULAR DISEASE

Although drug therapy is available for the treatment of cardiovascular disease, cardiovascular morbidity and mortality are poorly controlled throughout the world. Many of the pharmacological agents used to treat cardiovascular disease are expensive and therefore unavailable to poor segments of all societies. The effects of available drugs are transient or short lasting (usually shorter than 24 h), have adverse side effects and are not highly specific. Pharmacological therapy only partly mitigates cardiovascular complications. In addition, repeated doses and increased doses are required for long-term and chronic control of hypertension and related cardiovascular disease. Most cardiovascular diseases (hypertension, cardiac hypertrophy, etc) are multifactorial and multigenic; however, the drugs used for controlling the diseases are aimed at relatively few targets. Most patients with heart diseases are excluded from consideration for cardiac transplantation due to their medical complications and because of the shortage of donors. Clearly, it is imperative to develop new approaches to the treatment of cardiovascular disease. Gene therapy offers the possibility of producing longer-lasting and more specific effects based on the genetic design. Indeed, gene therapy opens a promising and novel avenue, although this new approach is in an early, experimental stage and, so far, has been short of clinical success. A recent study (26) demonstrated that postinfarction gene therapy aimed at suppressing tumour growth factor-beta signalling mitigated cardiac remodelling, attenuated cardiac fibrosis and heart failure, and improved infarct contraction. Thus, gene therapy may hold the promise for the treatment of post-MI heart failure and cardiac remodelling, which may be applicable during the subacute stage.

Considerations for gene therapy

The vector system: An effective and efficient vector system is essential for delivering therapeutic genes. Gene therapy uses a variety of gene transfer techniques. Some of the gene therapy approaches to cardiovascular disease have been recently

reviewed (27). Advantages and disadvantages of some of the viral- and nonviral-mediated gene delivery methods are discussed in the cited review. A vector is chosen according to its delivering efficiency, size, safety and stability. Of note, only a few of the currently available viral vectors achieve efficient, high-level transgene expression in postmitotic cells, such as cardiomyocytes. These include recombinant adenovirus (28), adeno-associated virus (29) and lentivirus (30). To illustrate, if one compares the vector with the computer hardware, then the software would be the therapeutic gene with its regulatory gene sequence. The latter gives scientists the opportunity to design an efficient therapeutic gene for a specific disease. The hypoxia-inducible, double plasmid system represents an exemplary gene engineering design (31): it can switch on protective genes to either depress the metabolic rate of or increase blood supply to ischemic tissue when tissue oxygen level decreases to a dangerous level. Adenovirus is not safe as a vector for human gene therapy (32) due to its propensity to initiate inflammation in the host. However, adenovirus is still widely used in the laboratory because of its high transduction efficiency, easy and fast packaging, transduction of both dividing and nondividing cells, and success in delivering genes to cells and animals. The ability to clone transgenes as large as 8 kb into replication-deficient adenoviral vectors has resulted in more efficient transgene expression in cardiac and vascular tissues.

The novel procedures for construction and packaging of adenoviruses with human endothelial nitric oxide synthase have been recently described by Wang et al (33). They demonstrated that human endothelial nitric oxide synthase can be expressed in both human aortic endothelial cells and rat heart cells, providing an interesting and promising approach for testing human gene expression in animals (34).

Recently, RNA interference is emerging as an important biological strategy for gene silencing and could potentially be a powerful tool for gene therapy. Small interfering RNA (siRNA)-mediated reduction in gene expression has been accomplished in mammalian cell culture by transfecting synthetic RNA oligonucleotides or plasmids, with the requirement that fragments be less than 30 base pairs to ensure specificity. Application of siRNA to *in vivo* gene silencing in mammalian tissues would require expression from intracellular transcription rather than transient transfection of double-stranded RNA. Viral-mediated delivery of siRNA has been shown to be effective in reducing expression of targeted genes specifically in various cell types, both *in vitro* and *in vivo* (35). Kasahara and Aoki (36) recently described technical details for gene silencing using adenoviral RNA interference in vascular smooth muscle cells and cardiomyocytes.

Vector delivery: The vector must be delivered, directly or indirectly, to the target organ or tissue, such as the heart. Employment of tissue-specific promoter is an ideal approach to achieve tissue-specific gene expression or inhibition. For example, the use of alpha-myosin heavy chain promoter will allow target gene expression and inhibition specifically in the heart. It has been reported that myosin light chain-2v can selectively deliver therapeutic genes to left ventricle while alpha-myosin heavy chain drives gene expression in the whole heart (11). Griscelli et al (37) and Jones and Koch (38) have recently described gene delivery approaches relevant to cardiovascular therapy.

Regulatory mechanism: A regulatory mechanism (sequence) should be incorporated into the vector to control

transgene expression or inhibition. This will allow the therapeutic gene to be regulated to achieve optimal treatment. The ideal promoter should be specific for the target cell type in the tissue and should be active for prolonged periods to maintain consistent levels of transgene expression. Thus, the promoter needs mechanisms to switch it on or off as required by pathological conditions. This hypothesis is being tested with the tetracycline transactivator system, by which a transgene can be switched on or off in the presence or absence of tetracycline (39). However, the tetracycline regulatory mechanism is leaky and therefore unsatisfactory. Obviously, an effective and reliable regulatory mechanism is needed before viral-mediated gene therapy can be used successfully in humans. This is a weak area that requires significant advances.

Reporter genes: Reporter genes should be employed to monitor real-time, tissue-specific transgene expression. Strategies for using conditional gene expression in myocardium have been described by Heine et al (40).

Finally, bridging the gap between these basic investigative studies and clinical gene therapy is a formidable but not insurmountable task. Experimental proof in rodents will need to be extended to large-animal models with clinical grade vectors and delivery systems to assess both efficacy and safety.

STEM CELL THERAPY FOR CARDIOVASCULAR DISEASES

The adult mammalian heart lacks the potential for effective regeneration. The infarcted myocardium is usually transformed into a noncontractile fibrous scar. This remodelling process leads to expansion of the initial infarcted area and dilation of the left ventricular lumen, resulting in congestive heart failure (41). Currently, no medication or procedure used clinically has shown efficacy in replacing the myocardial scar with functioning contractile tissue. A novel and attractive approach to the repair of damaged myocardium after MI is the use of stem cells (42-47). A recent report demonstrated that intramyocardial transplantation of human multipotent bone marrow (BM) cells after MI resulted in robust engraftment of transplanted cells, which exhibited colocalization with markers of cardiomyocyte, endothelial cell and smooth cell identity, consistent with differentiation of human BM stem cells into multiple lineages *in vivo* (48). In addition, up-regulation of paracrine factors, including angiogenic cytokines and anti-apoptotic factors, and proliferation of host cardiomyocytes and endothelial cells, were observed in the BM stem cell transplanted hearts. Thus, the favourable effect of BM stem cell transplantation after MI appears to be due to augmentation of proliferation and preservation of host myocardial tissues as well as differentiation of human BM stem cells for tissue regeneration and repair. The concept of stem cell therapy has already been introduced into the clinical setting, although the mechanistic underpinnings of stem cell therapy are still intensely debated. Preliminary clinical data indicate that stem cells have the potential to enhance myocardial perfusion or contractile performance in patients with acute MI and chronic heart failure (49).

Pluripotent stem cells are cells that have not taken on the identity of any specific cell type and are not yet committed to any dedicated function; they can divide indefinitely and may be induced to give rise to one or more specialized cell types. Murry et al (50) and Balsam et al (51) recently reported that

hematopoietic stem cells failed to transdifferentiate into cardiac myocytes in myocardial infarcts. The stem cells, however, developed into different blood cell types, despite being in the heart. Thus, for physicians, the use of stem cell therapy in treating cardiac muscle diseases remains a worthy, but perhaps long-term, goal. For scientists, how to effectively induce stem cells to differentiate into specific cell types is the key to the success of stem cell therapy. While there is proof of concept that transplantation of muscle cell progenitors may improve function after a heart attack, the task is to find the ideal source of cells. Therefore, it may be necessary to search naturally occurring, authentic cardiac progenitors, and to identify and dissect the signals that guide their migration and differentiation. Understanding of the biology of embryonic cardiac progenitors during development may offer new clues to cardiac stem cell therapy.

Because stem cell therapy is still in its experimental stage, there is a distinct advantage to evaluating many different types of stem cells, which will eventually lead to successful selection of optimal cells for cardiac stem cell therapy. It has been reported (52) that fetal heart cells may be optimal for heart-cell transplantation therapy because they graft easily, adopt the identity of an adult cardiac cell without fusion and are electrically coupled. Zeineddine et al (53) and Etzion et al (54) have recently employed novel protocols for myocardial repair using embryonic stem cells and fetal cardiac myocytes, respectively. However, the ethical controversy that surrounds their use in scientific research means that fetal stem cells may not be used in clinical studies, at least for the foreseeable future. Tang (55) has updated the technical procedures for using autologous mesenchymal stem cells and skeletal myoblasts (56) in myocardial repair. It has been reported that mesenchymal stem cells modified with Akt prevent remodelling and restore performance of infarcted hearts (57). Therefore, the use of stem cells genetically engineered with desired genes increases the potential of stem cell therapy. For example, a recent study (58) found that implantation of autologous BM mononuclear cells transfected with phVEGF165 can increase the number of surviving implanted cells and enhance functional improvement of infarcted hearts in rabbits.

CONCLUSIONS

Molecular cardiology has initiated new and exciting approaches to the study of cardiovascular diseases. These new approaches have contributed to traditional cardiology in many ways, including disease pathogenesis, diagnosis and treatment. Gene analysis and transgenic techniques have provided a new understanding of cardiovascular disease pathogenesis. Gene therapy and stem cell therapy have provided physicians and scientists with novel ideas for the treatment of heart diseases, although there is a lack of clinical success at this time. Therefore, there is an urgent demand for significant and rapid advances in molecular cardiology. These advances largely depend on the development of innovative technology in molecular and cellular biology.

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