Voordrachten

Clinical Perspectives of Molecular Cardiology

P. A. DOEVENDANS[1], V. van EMPEL[1], W. SPIERING[2,] R. van der ZEE[1]

Our insight into the development of cardiac disease is changing at a rapid pace. The tools have been developed by molecular biology to analyse the genetic cause of cardiac disease in humans. The knowledge of the genetic etiology not only helps to identify individuals at risk for inherited cardiac disease, it also increases our understanding of the pathophysiology and could potentially lead to new therapeutic strategies. During the Roche meeting an update was provided on the current techniques used to analyse the human genetic composition. New animal models are available to determine the effects of gene dosage. To illustrate the value of genetic modification in mice, models are discussed for atherosclerosis and hypertrophic cardiomyopathy. Furthermore, new methods are discussed to evaluate genetic variation in relation to atherosclerosis.

Basic principles of molecular biology
The power of the molecular approach for cardiovascular pathology and therapy, is based on the strict relation between DNA (carrying the genetic code), RNA (the messenger between nucleus and cytoplasm) and proteins with a specific physiologic function. In addition to the transcribed DNA, every gene has a regulatory sequence that controls the rate at which RNA is produced, determining the expression level of the gene. It is the regulatory part of a gene that has to be activated to start RNA production (transcription) (1). Which genes are active in different cell types depends on the interaction of their regulatory DNA with nuclear proteins. These nuclear proteins, the so-called transcription factors, can recognize specific regulatory DNA sequences and modulate the level of transcription. The nuclei of cardiomyocytes

contain specific transcription factors, which are different from for instance liver parenchymal cells, therefore other genes are being expressed in these two cell types (2-4).
All individuals have differences in their genetic composition. Most of these variations have no obvious effect as most of the DNA (90%) appears to have no function. Basepair changes positioned within exons alter the genetic code. As the triplet code is redundant some changes do not alter the amino acid sequence. Other base switches (missense mutations) can lead to one amino acid change, or truncation of the protein through the introduction of a stop codon. When one base is missing the triplet code shifts and a total new protein is the result (figure 1). Some of these mutations have been introduced into animals, to prove the causal relationship between the mutation and cardiovascular disease (5-7).

Hypertrophic and Dilated Cardiomyopathy
The term cardiomyoapthy indicates a disease originating in the muscle. Therefore, strictly spoken cardiac dysfunction after one or recurrent myocardial infarction, is not a cardiomyopathy as the cardiac damage is caused by vascular disease. Another common external cause for cardiac dysfunction is hypertension. Initially hypertension causes a generalized concentric hypertrophy later followed by left ventricular distension (hypertensive heart disease). Intrinsic cardiomyocyte dysfunction can lead to overt cardiac disease (cardiomyopathy). Depending on the genetic defect the disease can be recognized by marked excentric thickening of the left ventricular tissue including the interventricular septum and outflow tract. The disease can be so severe that the ventricular tissue at the base of the heart blocks the outflow of blood in the last part of the contraction phase. In addition, the disease is characterised by marked diastolic dysfunction based on impaired left ventricular compliance. Histologic sections reveal myofibrillar disarray which is a hallmark of the disease. In areas with a marked disarray the well defined and functional architecture of the muscular wall is disturbed and fiber direction seems to be random. The lack of fiber orientation and coordination leads to dysfunctional muscular regions. In addition, increased fibrosis can be demonstrated by specific immunohistochemical analysis. One of the first symptoms of the disease can be sudden cardiac

Dept. of Cardiology[1], Dept. of Internal Medicine[2], Academic Hospital Maastricht, Cardiovascular Research Institute Maastricht, The Netherlands

Address for correspondence: Pieter A. Doevendans, Dept. of Cardiology, Cardiovascular Research Institute Maastricht, PO Box 5800, 6202 AZ Maastricht.
E-mail: p.doevendans@cardio.azm.nl

Abbreviations: DCM: Dilated Cardiomyopathy; HCM: Hypertrophic Cardiomyopathy; HOCM: Hypertrophic Obstructive Cardiomyopathy
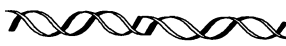
Point mutations

Normal	Missense	Stop	Frame shift

DNA

| ATG TCA GCC CAA CAG | ATG T**T**A GCC | ATG T**A**A GCC | ATG TC G CC C AA C AG C A |

Amino Acid

| Met-Ser-Ala-Gln-Gln | Met-***Leu***-Ala | Met-# | Met-Ser-**Pro**-Asn-Ser |
| Normal protein | Substitution | Truncated Protein | New protein |

| A | B | C | D |

Figure 1. The consequences of missense mutations. The normal situation of transcription and translation is shown in panel A. Panel B missense: the change of one base cytosine (C) at position 5 into thymidine (T) results in one amino-acid replacement (Serine-Leucine). Panel C stop codon: C to A (adenosine) change introduces a stop codon. This codon does not match with an amino acid and therefore translations stops. Panel D frame shift: the loss of A at position 6 leads to a shift in triplet codes, and therefore a new protein product.
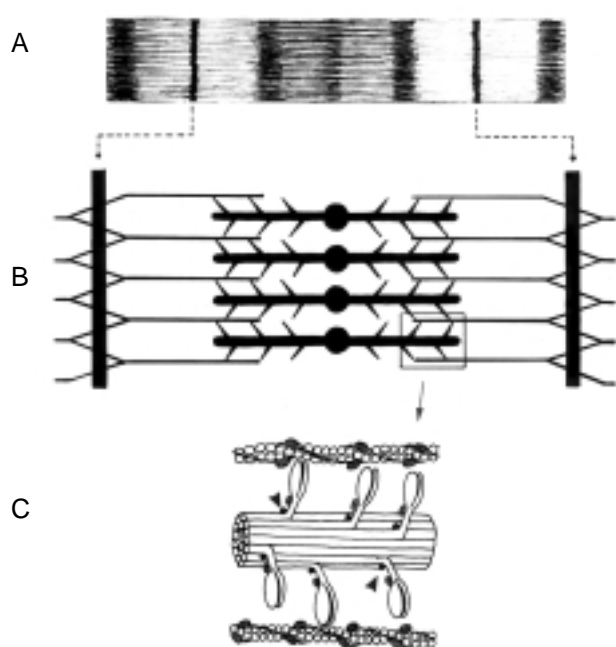

Figure 2. Schematic representation of striated muscle. A: the microscopic aspect of the cross striations and the various structural units composing the sarcomere in a relaxed state. B: schematic drawing showing the thick and the thin filaments and their position at the same magnification as panel A. C: proteins assembled in the sarcomere with the myosin cross-bridges interacting with actin. The position of the myosin light chains is indicated at the neck of the myosin molecules (arrow). Not shown is the position of the myosin binding protein C. This protein determines the location of the thick filaments by anchoring them to titin one of the sarcomere skeleton proteins.

death at adolescent age. More common symptoms are dyspnea, anginal complaints and dizziness. This disease has had many names in the past, but is now categorized as Hypertrophic CardioMyopathy (HCM) and in the presence of outflow tract obstruction as HOCM. Several of the genetic defects underlying HCM have been identified. In general, the inheritance pattern is autosomal dominant. Thus far mutations have been found in sarcomeric proteins, the proteins responsible for the formation of contractile elements. In most families mutations in the head of the beta myosin heavy chain (MHC) protein, located in the thick filament, cause the disease. The head of MHC molecule is the part of the protein domain that binds to actin in the thin filament (figure 2). However, the positions of the mutations vary and the functional implications have not been fully clarified yet. The risk of sudden death is linked to the site of mutation, without a clear relation to the morphological changes that can be found. This has also been reported for mutations in the troponin T gene. In these HCM families the hypertrophy can be mild, but with a poor prognosis as a high incidence of sudden death is often reported. The Troponin T gene is part of the protein complex composing the thin filament, and binds the troponin complex to tropomyosin. Additional mutations have been described in the myosin binding protein C (anchoring the thick filaments to titin), titin, troponin I, α-tropomyosin and the myosin light chains (8). Genetic manipulation in mice consists of either overexpression (more protein, transgenesis) or decreased expression ('knock-out', less protein) of the gene of interest. The transgenic approach involves

injection of constructs into the pronucleus of fertilized oocytes. This technique results in the introduction of one or more copies of the transgene into the genome at random sites. Therefore, the level of over-expression of the new insert is unpredictable and varies between different lines. In contrast, to generate knock-out mice homologous recombination is used to destroy one target allele. By crossing heterozygote animals, homozygote knock outs can be generated in which the target gene is completely absent. The causal relation of the mutations leading to hypertrophic cardiomyopathy was verified by creating transgenic mouse models. In mouse the α-MHC is the predominated isoform expressed in adult heart. The head of the MHC molecule in both isoforms (α and β) is highly conserved in mouse and man. Therefore the human β-MHC mutation was introduced in the a MHC gene and inserted into the mouse genome. The transgenic mice developed the full clinical picture of HCM, with asymmetric hypertrophy, left atrial dilatation and including sudden death (9). To assess functional parameters in mice, the tools to perform hemodynamic studies had to be miniaturized. The small size and high heart rate of the mouse pose technical challenges in measuring functional parameters by invasive hemodynamic investigation, two-dimensional and Doppler-echocardiography and magnetic resonance imaging. However, the ease of genetic modification and the short reproductive cycle allow unique opportunities to investigate functional consequences of genetic changes. In the mouse left ventricular pressures and cardiac output can be determined. Hemodynamic studies in the HCM mouse model revealed an impaired relaxation of the left ventricle in early stages (9).

Atherosclerosis

The number one cause of mortality in our Western society is coronary artery disease. Atherosclerosis is the underlying disease, that can be complicated by acute thrombotic events leading to vascular occlusion and myocardial infarction. Also in this disease, genetics play an important role although the influence of the environment is crucial in many individuals. The contribution by genetic factors varies from 100% in patients, who have no receptor for low density lipoproteins or mutations in the ApoE3 gene, to approximately 2-3% in the presence of defined lipoprotein lipase LPL polymorphisms (10-11). In patients with a variation in the LPL gene, the environmental factors determine the severity and outcome of the disease. In many patients developing coronary artery disease a genetic cause seems likely from the family history, although no abnormal chemical parameters can be identified. The effect of mutations in genes coding for lipoproteins was demonstrated for the ApoE3*Leiden gene. In a large family with early onset of cardiovascular disease, leading to myocardial infarction, cerebrovascular accidents and death, the diagnosis of dysbetalipoproteinemia was made. The genetic defect appeared to be localized within the ApoE gene. The ApoE3*Leiden variant turned out to contain a point mutation and an insertion of 21 base

pairs leading to a 7 amino acid repeat. To determine the causal role of this human genetic variant for the etiology of atherosclerosis, the human gene was introduced into the mouse genome. For the ApoE3 gene also knock out mice have been generated and studied (12). These animals developed atherosclerosis even on a normal diet. Another group made a transgenic line using the defective human ApoE3*Leiden gene (13-14). So in addition to the mouse ApoE gene, the transgenic mouse strain contained copies of the ApoE3*Leiden gene. In contrast to normal (wild type) mice, these mice are susceptible to atherosclerosis when fed on a high fat diet. The histology and complexity of the atherosclerotic lesions in these mice matches human disease (15-16). However, in the ApoE3*Leiden mice, no acute vascular events have been documented leading to myocardial or cerebral infarcts. A solid fibrous cap prevents plaque rupture in this model. The ApoE3*Leiden mice demonstrate one aspect of the power of genetic analysis. The identification of the causal human monogenetic defect was confirmed in the transgenic experiment.

Multigenic cause of atherosclerosis

As mentioned above in the majority of patients the disease can not be explained by a mutation in a single gene. It is likely that unfavorable polymorphisms in various genes predispose to atherosclerosis. A polymorphism indicates a genetic variation which is found in more than 1% of the general population. The polymorphism in itself is not necessarily associated with altered protein function. Very often the polymorphisms are associated with a different protein level. If the polymorphisms involve lipoprotein levels, coagulation factors and inflammatory genes, the combination of polymorphisms could contribute to atheresclerosis (multiple hit theory). As atherosclerosis is a complex disease many genes could enhance the genetic susceptibility of an individual. It has become clear that many organs, cell types and circulating factors can play a role in atherosclerotic lesion formation. To tackle this question, more advanced techniques have to be developed which determine multiple polymorphisms in different genes simultaneously (17-18). Furthermore, large populations have to be studied to unravel the role of all the potentiating genetic combinations. New molecular techniques could be helpful for this approach. One is the multiplex PCR reaction, using preloaded membranes to identify > 60 polymorphisms in one reaction. An alternative is the use of microarray or chip technology to study thousands of sites overnight. The department of Molecular Cardiology Maastricht is currently testing a new kit developed by Roche Diagnostics using the multiplex PCR and thus far the results match previously analyzed DNA samples for the Insertion(I)/ Deletion(D) polymorphism of the Angiotensin Converting Enzyme (ACE) gene (n=20). The I/D polymorphism is one of the most frequently analyzed genetic variations in man. The polymorphism is based on the insertion of 287 basepairs in intron 16 of the ACE gene and associated with the level of circulating ACE enzyme. In small studies dramatic associations

Table 1. Multiplex PCR for polymorphism detection

Class of genes	Polymorphisms
Lipoproteins	24
Coagulation	10
Inflammation: Cytokines	7
Metabolism	7
Endothelial Function	7
Receptors and channels	6
Natriuretic peptides	2
Total	63

have been found that were never confirmed in larger cohorts (19-21). The results of the multiplex PCR kit are completely reproducible using identical samples three times (n=15). Several groups of genes involved in the etiology of atherosclerosis are included in this biochemical assay (see table 1).

Conclusion

Molecular techniques have improved our understanding of the pathophysiological processes underlying cardiovascular disease and have proven to be an invaluable tool for the identification of genetic causes of human disease. New techniques are within reach of every clinical center to unravel multiple genetic factors that contribute to the predisposition for coronary artery disease.

References

1. Doevendans PA, Gilst WH van, Laarse A van der, Bilsen M van. Molecular cardiology part I: Gene transcription in the cardiovascular system. Cardiologie 1997; 4: 221-229.
2. Barton PJR. Molecular biology and the heart. In: 1990-91 annual of Cardiac Surgery. Yacoub M, Pepper J, eds. 1991. Current Science, London.
3. Swynghedauw B, Moalic JM, Bourgeois F, Barrieux A. An introduction to the jargon of molecular biology. Part II. Cardiovasc Res 1993; 27: 1566-1575.
4. Swynghedauw B, Barrieux A. An introduction to the jargon of molecular biology: Part I. Cardiovasc Res 1993; 27:1414-1420.
5. Windt L de, Wilde AAM, Doevendans PA. Molecular Cardiology part 5: Animal models in molecular cardiovascular research. Cardiologie 1998; 5: 327-335.
6. Doevendans PA, Hunter JJ, Lembo G, Wollert KC, Chien KR. Strategies for studying cardiovascular diseases in transgenic mice and gene-targeted mice. In: Strategies in transgenic animal science. Monastersky GM, Robl JM, eds. 1995. American Society For Microbiology, Washington.
7. Doevendans PA, Daemen M, Muinck E de, Smits J. Cardiovascular phenotyping in mice. Cardiovas Res 1998; 39: 34-49.
8. Spirito P, Seidman CE, McKenna WJ, Maron BJ. The management of hypertrophic cardiomyopathy. N Engl J Med 1997; 336: 775-785.
9. Geisterfer Lowrance AA, Christe M, Conner DA, Ingwall JS, Schoen FJ, Seidman CE, Seidman JG. A mouse model of familial hypertrophic cardiomyopathy. Science 1995; 272: 731-734.
10. Goldstein JL, Brown MS. The LDL receptor locus and the genetics of familial hypercholesterolemia. Annu Rev Genet 1979; 13: 259-289.
11. Jukema JW, Boven AJ van, Groenemeijer B, Zwinderman AH, Reiber JH, Bruschke AV, Henneman JA, Molhoek GP, Bruin T, Jansen H, Gagne E, Hayden MR, Kastelein JJ. The Asp9 Asn mutation in the lipoprotein lipase gene is associated with increased progression of coronary atherosclerosis. REGRESS Study Group, Interuniversity Cardiology Institute, Utrecht, The Netherlands. Regression Growth Evaluation Statin Study. Circulation 1996; 94: 1913-1918.
12. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell 1992; 71: 343-353.
13. Maagdenberg AM van den, Hofker MH, Krimpenfort PJ, Bruyn I de, Vlijmen B van, Boom H van der, Havekes LM, Frants RR. Transgenic mice carrying the apolipoprotein E3-Leiden gene exhibit hyperlipoproteinemia. J Biol Chem 1993; 268: 10540-10545.
14. Dijk KW van, Vlijmen BJ van, Hof HB van 't, Zee A van der, Santamarina-Fojo S, Berkel van TJ, Havekes LM, Hofker MH. In LDL receptor-deficient mice, catabolism of remnant lipoproteins requires a high level of apoE but is inhibited by excess apoE. J Lipid Res 1999; 40: 336-344.
15. Lutgens E, Daemen M, Kockx M, Doevendans PA, Hofker M, Havekes L, Wellens HJ, Muinck E de. Atherosclerosis in APOE*3 Leiden Transgenic Mice: From Proliferative to Atheromatous Stage. Circulation 1999; 99: 276-283.
16. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1995; 92: 1355-1374.
17. Funke H, Assmann G. Strategies for the assessment of genetic coronary artery disease risk. Curr Opin Lipidol 1999; 10: 285-291.
18. Rosenthal N, Schwartz,RS. In search of perverse polymorphisms. N Engl J Med 1998; 338: 122-124.
19. Kuznetsova T, Staessen JA, Wang JG, Gasowski J, Nikitin Y, Ryabikov A, Fagard R. Antihypertensive treatment modulates the association between the D/I ACE gene polymorphism and left ventricular hypertrophy: a meta-analysis. J Hum Hypertens 2000; 14: 447-454.
20. Linhart A, Sedlacek K, Jachymova M, Jindra A, Beran S, Vondracek V, Heller S, Horky K. Lack of association of angiotensin-converting enzyme and angiotensinogen genes polymorphisms with left ventricular structure in young normotensive men. Blood Press 2000; 9: 47-51.
21. Keavney B, McKenzie C, Parish S, Palmer A, Clark S, Youngman L, Delepine M, Lathrop M, Peto R, Collins R. Large-scale test of hypothesised associations between the angiotensin-converting-enzyme insertion/deletion polymorphism and myocardial infarction in about 5000 cases and 6000 controls. International Studies of Infarct Survival (ISIS) Collaborators. Lancet 2000; 355: 434-442.