Genetic, clinical and experimental aspects of restenosis : a biomedical perspective

Monraats, P.S.

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General introduction and outline of the thesis

Atherosclerosis is a type of arteriosclerosis. It comes from the Greek words athe ro (meaning gruel or paste) and sclerosis (hardness). Atherosclerosis is a disease of the arterial intima leading to the formation of fibrous (atheromatous) plaques and to stenosis of the lumen. It involves the proliferation of smooth muscle cells and the accumulation of lipids. Atherosclerosis affects large and medium-sized arteries. The type of artery and the localisation of the plaque vary between per sons. In general, atherosclerosis is a slow, progressive disease that may start in childhood. In some people this disease progresses rapidly in their fourth decade. In others atherosclerosis becomes symptomatic when they are in their fifties or sixties. Atherosclerosis and its complications represent the dominant cause of mortality and morbidity in the Western world.[1]

Modern therapy of atherosclerotic disease lowers plasma lipid level by statin treatment. However, when this therapy is insufficient, or for a patient with an acute coronary event, the therapy of choice has become Percutaneous Coronary Intervention (PCI). PCI is a safe and effective way to unblock coronary arteries. During this procedure, a catheter is inserted into an artery and guided forward into the coronary arteries of the heart. There, blocked arteries can be opened with a balloon positioned at the tip of the catheter.[2] Initially, angioplasty was performed with balloon catheters only, but technical advances have been made and improved patient outcome has been achieved by placement of small metal lic spring-like devices called "stents" at the site of previous blockage.[3;4] The implanted stent serves as a scaffold that keeps the artery open. Angioplasty and stenting techniques are widely used around the world and provide an alternative option to medical therapy and bypass surgery for improving blood flow to the heart muscle. However, one of the main drawbacks of PCI is the occurrence of restenosis.[5;6]

Pathogenesis of restenosis

Restenosis is commonly defined as the re-narrowing of the lumen to greater than 50% occlusion angiographically after PCI usually within three to six months.[7] It is thought that the mechanism of this phenomenon is not mainly the progres sion of coronary artery disease, but rather the response of the body's immune system to the "injury" induced by angioplasty. At this point a repeat procedure may be needed. Compared to balloon angioplasty (BA) alone, having a chance of restenosis of 40%, stents reduce the chance of in-stent restenosis to 10-25%.[4;8;9] Therefore, the majority of patients having angioplasty today are treated with stents.

Three distinct processes are involved in the pathogenesis of restenosis: vessel recoil and neointimal proliferation sometimes followed by early thrombus for

mation. The relative contribution of each of these depends on the type of injury. About three quarters of lumen loss after BA is the result of vessel recoil, and the rest is the result of neointimal proliferation.[10] Coronary stenting virtually eliminates vessel recoil, and restenosis is largely the result of neointimal prolif eration. When an artery is injured, deposition of platelets, leukocyte infiltration, proliferation of smooth muscle cells, deposition of extracellular matrix, and re endotheliazation occur. Growth factors and cytokines released by the blood cells involved stimulate the migration, growth, and multiplication of smooth muscle cells.[7;11;12] A few years ago, a breakthrough in the prevention of restenosis was introduced by a new generation of "drug-eluting" stents. These stents release a particular drug that prevents scar tissue growth in the artery in which the stent is placed, and therefore reduce the occurrence of restenosis. Recent data have demonstrated that patients treated with drug-eluting stents have decreased inci dence of restenosis compared to those who received bare metal stents.[13]

Risk factors for restenosis

Identification of patients at increased risk for restenosis may improve stratifica tion of patients to individually tailored treatment. A number of clinical charac teristics, such as advanced age, history of diabetes and several plasma markers, as well as angiographic details, such as the severity of coronary lesions, lesion length before the procedure, and minimal lumen diameter after the procedure have been shown to be associated with an elevated risk of restenosis after PCI.[14-18] Thus far, however, these clinical factors have not been consistently found in different studies. Therefore, patients cannot be stratified with regard of risk for coronary restenosis based only upon clinical or procedural risk factors. There is evidence that genetic factors explain part of the excessive risk of restenosis after PCI independently of conventional clinical variables. For the same level of environmental risk, two individuals may have different probabilities of develop ing a cardiovascular event.[19] Furthermore, in patients with multivessel disease, the incidence of restenosis of a second lesion was 2.5 times higher if PCI of the first lesion had led to restenosis, even after adjustments for known patient-re lated risk factors, including diabetes and hypertension.[20] However, the charac terisation and identification of DNA variants (polymorphisms) responsible for this variation in individual susceptibility are complicated by the many factors involved in restenosis.

Genetic predisposition to restenosis

Today, a person's genetic background is considered in every aspect of clinical medicine, ranging from susceptibility to diseases, pathogenesis, and clinical out come to diversity in responses to drug treatment (pharmacogenomics). The new panoramic look at the human genome has stimulated a massive search for clini cally relevant genomic information, including single nucleotide polymorphisms (SNPs) that consist of substitutions of one nucleotide for another in a DNA se quence. Individual genomes are 99.9 percent identical, with only 0.1 percent of the genome showing polymorphisms.[21;22] About 2 to 3 million SNPs have been found in exonic, intronic, regulatory, and intergenic regions. Almost all genes contain SNPs, but only a minority may have functional consequences because they either affect the expression level of a protein or predict an alteration of the amino acid sequence. Complexity increases at the protein level, since one human gene may produce up to five different proteins as a result of alternative splicing. Posttranslational modifications, such as assembly or glycosylation, further in crease the diversity of proteins. Furthermore, environmental and other genetic factors may alter the phenotype that results from genetic abnormalities.

One of the main problems in the field of genetic association studies is the poor reproducibility.[23] In addition to true biological effects that influence the rela tionship of interest, a number of methodological and epidemiological factors may distort the results of genetic association studies, including definitions of patients and controls, population heterogeneity, and limited statistical power. These limitations have cast doubts on this type of study,[24-27] and several bio medical journals have even adopted a policy of not publishing the results of as sociation studies related to complex diseases. These difficulties, however, should be weighed against the potential benefits of genetic research. Because genetic predisposition does not fluctuate as do, for instance, plasma levels of a given risk factor, risk prediction based on genetic profiling would be a very effective approach. In addition, the studies of protein risk factors independent of their genetic determinants will likely result in an incomplete understanding of the pathophysiology. Recently, recommendations have been made for the design of future genetic association studies. The first recommendation concerned the ad equacy of statistical power; the studies should have enough power to detect a relative risk of 1.25 or more. Recent evidence suggests that the large majority of genetic association studies performed thus far have been underpowered to de tect the difference under study. A second important recommendation was that an observed genotype-disease association should be accompanied by supporting evidence from literature or from theoretical points of view for candidate vari ants to be involved in the disease. Furthermore, for each selected gene locus only

functional variants (i.e. variants that alter an amino acid or a transcription factor-binding element in a promoter region) should be included. Finally, the genotype must give a predictive value in carriers over and above established risk factors and the final data set should show no significant evidence for heterogeneity of risk effect.[28]

Aim of this thesis

The GENetic DEterminants of Restenosis (GENDER) project was designed to study the association between various gene polymorphisms and clinical reste nosis. Therefore, the aim of this thesis was to examine which polymorphisms, with its main focus on polymorphisms in genes involved in the inflammatory response, may contribute to the stratification of patients who are at increased risk of developing of restenosis. In addition, we also analysed different clinical factors.

GENDER study

The GENDER project is a multicenter follow-up study. Patients were eligible for inclusion if they were successfully treated for stable angina, non-ST-eleva tion acute coronary syndromes, or silent ischemia by PCI. Patients treated for acute ST elevation myocardial infarction (MI) were excluded. All patients were treated in four referral centers for interventional cardiology in the Netherlands (Academic Medical Center Amsterdam, University Medical Center Groningen, Leiden University Medical Center and Academic Hospital Maastricht). The overall inclusion period lasted from March 1999 until June 2001, with inclusion intervals varying between the centers in order to include equal numbers of pa tients per center. In total, 3,104 consecutive patients were included in this pro spective multicenter follow-up study.

The angiographic follow-up substudy

In addition to the clinical follow-up 480 patients also had a follow-up angiogra phy at 6 months after PCI. Although the clinical endpoint depicts the clinical relevance of any risk factor more clearly, angiographic measurements quantify the restenosis process on a continuous scale. The angiograms have been studied in a Core laboratory to ensure standardized analysis (Heartcore, Leiden)

The protein substudy

In any genetic association study the ultimate proof of causality is the confirma tion of an association with altered levels of gene-related proteins.[29;30] However, to predict the development of restenosis in the individual patient plasma deter

minations may have no additive value, since basal (pre-PCI) plasma measure ments of the gene product are not likely to reflect the genetically determined differences in reaction to a trauma such as PCI. However, to study the effects of several polymorphisms in the development of restenosis, plasma levels of se lected gene products were measured by enzyme-linked immunosorbent assay in lipopolysaccharide-stimulated whole blood in a subpopulation of patients (N=69).

Mouse model of restenosis

To increase the understanding of the process of restenosis a mouse model can be used. In our study we made use of a well-defined mouse model. Diet-dependent ApoE*3-Leiden transgenic mice aged 8-10 weeks, weighing 25-30g, were fed a western-type diet 3 weeks prior to surgery, which diet was continued after sur gery in order to maintain stable plasma cholesterol levels. The institutional com mittee on animal welfare of TNO-PG had approved the animal experiments. After 3 weeks on diet, mice were anaesthetized. The right femoral artery was prepared free and a 3 mm non-constricting polyethylene cuff (Portex, 0.40 mm inner diameter, 0.80 mm outer diameter) was placed loosely around it.
Animals were sacrificed at different time points after surgery (6, 24, 48 hours and 7 days after cuff implantaion), 4 mice for each time point. Both cuffed right femoral artery and untreated left femoral artery were prepared free, harvested and snap frozen. RNA was isolated and cDNA was synthetised using Ready-To-Go RT-PCR beads.[31-33]
Perivascular cuff-placement result initially in adventitial injury, whereas PCI in patients primarily results in intimal injury. It is not certain to what extent these types of vascular injury differ in their reaction regarding vascular activation and the resulting intimal hyperplasia. However, the observation that local delivery of anti-restenotic agents using a drug-eluting polymer cuff in the mouse model inhibits neointima formation demonstrates that the model is useful to improve our understanding of the process of restenosis.[34]

Focus of this thesis

Inflammatory responsiveness, which is highly genetically determined, plays a pivotal role in the process of restenosis.[35-38] Many studies have demonstrated genetic influences upon the inflammatory response of an individual.[39;40] There fore, it is plausible that differences in genetic make-up of inflammatory genes between individuals may explain part of the risk of restenosis. Indeed, associa tion studies have identified several candidate genes polymorphisms of which may predispose to restenosis, such as polymorphisms of the genes; stromely

sin-1, Interleukin-6, E-selectin, CD18, CD14 and Interleukin-1 receptor.[35;36;41] Therefore, the main focus of this thesis is to examine genes and their polymor phisms involved in inflammatory pathways in patients of the GENDER popula tion. We started by using validated multilocus genotyping assays to test several markers of inflammation and cardiovascular disease, [42;43] in a large set of genes. Next, we continued genotyping polymorphisms with varying allele frequencies and distributed through the promoter, coding and 3'-untranslated regions of sev eral candidate genes. Genes having polymorphisms that are significantly associ ated with restenosis were studied in the mouse model of restenosis, particularly to find out whether these genes were up or downregulated during the develop ment of neointima. To further determine the functional effect of several poly morphisms examined, we performed whole-blood stimulation and tested effects on gene expression.

Outline of this thesis

This thesis focuses on clinical restenosis after PCI. Although we describe clini cal factors involved in the development of restenosis, we focus mainly on genetic factors associated with restenosis. Chapter 2 reviews the genetic factors related to restenosis at the time we started genotyping. In chapter 3 we describe the GENDER population and the clinical factors associated with restenosis. Since diabetes is the strongest clinical risk factor of restenosis and is one of the five elements defining metabolic syndrome, we examined whether metabolic syn drome predicts the occurrence of restenosis in chapter 4. Chapter 5 presents the results of a gene-array study, in which we tested 48 polymorphisms in 34 genes involved in inflammation. In chapter 6, we present a promoter polymorphism of the fibrinogen gene, and in this study we also determined fibrinogen levels in the plasma of a subpopulation of patients. Polymorphisms in the lipoprotein lipase (LPL) gene have been extensively described and are known to have an effect on LPL activity. In chapter 7 we examined four different polymorphisms of the LPL-gene and their relation to restenosis. The goal of chapter 8 was to investi gate the role of the TNFα gene in the restenosis process. TNFα is one of the key regulators of the inflammatory response and may therefore play an impor tant role in the restenosis process. Chapter 9 explores the relationship between genetic variants in the caspase-1, PTPN22 and IL-1r genes and restenosis, which have been identified as important mediators in the inflammatory response and for caspase-1 also in apoptosis. Finally, since little is known about the involve ment of anti-inflammatory cytokines in restenosis, chapter 10 examines the role of four different IL-10 polymorphisms in the development of restenosis. IL-10 is an important immunosuppressor cytokine, involved in the regulation of many aspects of immune responses.

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