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

Monraats, P.S.

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Interleukin 10: a new risk marker for the development of restenosis after per cutaneous coronary intervention

Pascalle S. Monraats, Fina A.S. Kurreeman, Douwe Pons, Varsha D.K.D. Sew gobind, Florentine R. de Vries, Aeilko H. Zwinderman, Moniek P.M. de Maat, Pieter A. Doevendans, Robbert J. de Winter, René A. Tio, Johannes Walten berger, Tom W.J. Huizinga, Daniël Eefting, Paul H.A. Quax, Rune R. Frants, Arnoud van der Laarse, Ernst E. van der Wall, J. Wouter Jukema

submitted

Abstract

Genetic factors appear to be important in the process of restenosis after PCI, as well as in inflammation, a pivotal factor in restenosis. An important mediator in the inflammatory response is IL-10. Our aim was to study whether genetic vari ants in IL-10 predispose to the risk of restenosis. The GENetic DEterminants of Restenosis (GENDER)-project, a multicenter follow-up study, included 3,104 patients treated with successful PCI. TVR, either by PCI or CABG, was chosen as primary endpoint.

Genotyping of the -2849G/A, -1082G/A, -592C/A and +4259A/G polymorphisms of the IL-10 gene was performed by MassArray platform. After adjusting for clinical variables three polymorphisms significantly increased the risk of reste nosis (-2849AA: RR; 1.7, 95%CI; 1.2-2.5, -1082AA: RR; 1.4, 95%CI; 1.1-1.8, and +4259GG: RR; 2.0, 95%CI; 1.4-2.8). To further exclude possible involvement of neighboring genes due to LD in the IL-10 locus, additional polymorphisms were genotyped. The results reveal that association of the IL-10 gene with restenosis is independent of flanking genes.

Our findings demonstrate that IL-10 is associated with restenosis and therefore support the hypothesis that also anti-inflammatory genes may be involved in de veloping restenosis. Furthermore, they contribute to unravelling the restenotic process and may provide a new targeting gene for drug-eluting stents.

Introduction

An important limitation of the treatment of atherosclerotic lesions by percuta neous coronary intervention (PCI) is the occurrence of restenosis. [1;2] Inflam matory responsiveness, resulting in neointima formation, plays a pivotal role in the development of restenosis.[3-5] Several inflammatory genes have already been reported to be associated with the development of restenosis.[6;7] However, little is known about the involvement of anti-inflammatory cytokines, although they seem logic candidate genes in the process of restenosis. Interleukin 10 (IL-10) is one of these anti-inflammatory genes. It is an important immunosuppressor cytokine, involved in the regulation of many aspects of immune responses. Its effects are directed mainly against functions of mononuclear cells, T lympho cytes, and polymorphonuclear leukocytes. Furthermore, IL-10 plays a role in inhibition of cell adhesion molecules, monocyte chemotactic protein-1, tissue factor, fibrinogen, matrix metalloproteinase-9, T-lymphocyte granulocyte-mac rophage colony-stimulation factor, inducible nitric oxide synthase, and smooth muscle cell proliferation.[7-11] Several of these factors have been demonstrated to be involved in the restenotic process.[7-11] The interindividual difference among individuals in their ability to produce IL-10 appears to have a genetic origin. [12-14] The heritability of the endotoxin-induced IL-10 production has been estimated to be 74% in studies on monozygotic or dizygotic twins and nonrelated individu als.[15] The gene encoding IL-10 contains variable sites (polymorphisms and mic rosattellite markers) that have previously been associated with the level of IL-10 produced indicating that they may be associated with different responsiveness to regulatory signals. The aim of this study was to assess whether four different functional polymorphisms (3 in the promoter, 1 in the 3'UTR) in the IL-10 gene are related to the risk of developing restenosis after PCI.

Materials and Methods

GENetic DEterminants of Restenosis (GENDER) project

Study design

The present study population has been described previously.[16] In brief, the GE Netic DEterminants of Restenosis (GENDER) project was designed to study the association between various gene polymorphisms and clinical restenosis.

Patients eligible for inclusion were treated successfully for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia by PCI in four out of 13 referral centers for interventional cardiology in the Netherlands. Patients treated for acute ST elevation myocardial infarction (MI) were excluded. Also excluded from analysis were patients suffering from events occurring within one month after PCI, since these events were attributable predominantly to sub-acute stent thrombosis or occluding dissections, rather than to restenosis.

PCI procedure

Experienced operators, using a radial or femoral approach, performed standard angioplasty and stent placement. Before the procedure, patients received aspirin 300 mg and heparin 7500 IU. The use of intracoronary stents and additional medication, such as glycoprotein IIb/IIIa inhibitors, were carried out at dis cretion of the operator. In case a stent was implanted, patients received either ticlopidin or clopidogrel for at least one month following the procedure depend ing on local practice. During the study, no drug-eluting stents were used.

Follow-up and study endpoints

Follow-up lasted for at least nine months, except when a coronary event oc curred. Patients were either seen in outpatient clinics or contacted by telephone. Target vessel revascularization (TVR), either by PCI or coronary artery bypass grafting (CABG), was designated the primary endpoint, since it is considered most relevant by regulatory agencies. An independent clinical events committee evaluated the clinical events.

The study protocol meets the criteria of the Declaration of Helsinki and was approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from all participating patients prior to the PCI procedure.

Genetic methodology

Blood was collected in EDTA tubes at baseline and genomic DNA was extracted following standard procedures. In this population we determined genotypes of the following polymorphisms in the IL-10 gene: -2849G/A (rs6703630), -1082G/A (rs1800896), -592C/A (rs1800872) and +4259A/G (rs3024498). These polymor phisms were selected from literature, and databases on the web, criteria used were frequency of the rare allele and a possible functional effect.[7;9-11;17] To assay these polymorphisms, we used a MassArray platform according to manufactur er's protocols. Two multiplex assays were designed using Assay designer software (Sequenom, Hamburg, Germany). After PCR a primer extension reaction was

performed to introduce mass-differences between alleles and, after removing salts by adding a resin, ~15 nl of the product was spotted onto a target chip with 384 patches containing matrix. Mass differences were detected using an Auto flex (Bruker, Wormer, Netherlands) matrix-assisted laser desorption\ionisation time-of-flight mass spectrometry (MALDI-TOF) and genotypes were assigned real-time using Typer 3.1 software (Sequenom). As quality controls, 5-10% of the samples were genotyped in duplicate. No inconsistencies were observed. Cluster plots were made of the signals from the low and the high mass allele. Two inde pendent researchers carried out scoring. Disagreements or vaguely positioned dots produced by Genotyper 3.0 (Sequenom) were excluded from analysis.

Statistical analysis

Deviations of the genotype distribution from that expected for a population in Hardy-Weinberg equilibrium was tested using the Chi-squared test with one de gree of freedom. Allele frequencies were determined by gene counting, the 95% confidence intervals of the allele frequencies were calculated from sample allele frequencies, based on the approximation of the binominal and normal distribu tions in large sample sizes.

Continuous variables are expressed as mean ± standard deviation and were com pared by means of the unpaired, two-sided *t* test. Discrete variables are expressed as counts or percentages and were compared with the Chi-squared test. In the first stage, the association between the IL-10 polymorphisms and TVR was as sessed using the Cox proportional regression model under a co-dominant ge netic model. No adjustments for covariates were performed at this stage so that we could assess their possible involvement in the causal pathway.

All polymorphisms were also assessed using dominant and recessive models, and the model with the lowest Akaike information criteria was used in multivariable regression analysis. [18] Multivariable regression analysis of the TVR risk was per formed with all IL-10 polymorphisms, using a stepwise backward selection algo rithm. In the final step clinical variables associated with TVR, also including age and gender, were entered into the regression model. The IL-10 polymorphisms were combined into haplotypes and the effect of haplotypes on restenosis risk was estimated according to the methods developed by Tanck et al.[19] Evalua tion of the neighboring genes of the IL-10 locus was performed by genotyping polymorphisms in the MAPKAPK2, IL-19, IL-20 and IL-24 genes in a panel of healthy individuals (N=60). Haploview software (http://www.broad.mit.edu/mpg/haploview) was used to perform LD calculations. Statistical analysis was carried out using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered statistically significant.

Results

A total of 3,146 patients had a complete follow-up (99.3%) with a median dura tion of 9.6 months (interquartile range 3.9 months). Out of 3,146 patients 42 had an event in the first 30 days. These patients were excluded from further analysis, according to the protocol. Baseline characteristics of the population are shown in Table 1.

Table 1. Demographic, clinical and lesion characteristics of 3,104 patients with and without TVR

	Patients with TVR (n=304)	Patients without TVR (n=2,800)	Total (n=3,104)
Age (years)	61.7 ± 10.1	62.2 ± 10.8	62.1 ± 10.7
BMI (kg.m^{-2})	26.9 ± 3.7	27.0 ± 3.9	27.0 ± 3.9
Male sex	220 (72.4%)	1,996 (71.3%)	2,216 (71.4 %)
Diabetes	63 (20.7%)	390 (13.9%)	453 (14.6%)
Hypercholesterolemia	188 (61.8%)	1,702 (60.8%)	1,890 (60.9%)
Hypertension	138 (45.4%)	1,121 (40.0%)	1,259 (40.6%)
Current smoker	62 (20.4%)	700 (25.0%)	762 (24.5%)
Family history of MI	121 (39.8%)	977 (34.9%)	1,098 (35.4%)
Previous MI	109 (35.9%)	1,130 (40.4%)	1,239 (39.9%)
Previous PCI	64 (21.1%)	493 (17.6%)	557 (17.9%)
Previous CABG	36 (11.8%)	340 (12.1%)	376 (12.1%)
Stable angina	198 (65.1%)	1,881 (67.2%)	2,079 (67.0%)
Multivessel disease	148 (48.7%)	1,284 (45.9%)	1,432 (46.1%)
Peripheral vessel disease	12 (3.9%)	92 (3.3%)	104 (3.4%)
Lipid lowering medication	171 (56.3%)	1,516 (54.1%)	1,687 (54.3%)
Restenotic lesions	27 (8.9%)	181 (6.5%)	208 (6.7%)
Total occlusions	56 (18.4%)	372 (13.3%)	428 (13.8%)
Type C lesion	94 (30.9%)	708 (25.3%)	802 (25.8%)
Proximal LAD	70 (23.0%)	619 (22.1%)	689 (22.2%)
RCX	75 (24.7%)	764 (27.3%)	839 (27.0%)
Residual stenosis >20%	51 (16.8%)	299 (10.7%)	350 (11.3%)
Stent length (mm)	10.3 (0-82)	13.0 (0-93)	15 (0-146)

BMI: body mass index, MI: myocardial infarction, CABG, coronary artery bypass grafting, LAD: left anterior descending branch of the left coronary artery, RCX: circumflex branch of the left coronary artery. Age is mean ± SD; other variables are percentage of patients

Genotyping was successful in 2,874 patients for the -2849G/A polymorphism, in 2,740 patients for the -1082G/A polymorphism, in 2,873 patients for the -592C/A polymorphism and in 2,865 patients for the +4259A/G polymorphism. Allele frequencies were: 0.72/0.28, 0.51/0.49, 0.76/0.24, and 0.73/0.27, respectively. The results of the remaining patients are missing due to lack of DNA or inconclusive genotyping. Patients who could not be genotyped did not differ in any charac teristic from those who could be genotyped. The distributions of the genotypes are shown in Table 2. All polymorphisms showed no significant deviation from Hardy Weinberg equilibrium (p>0.05), except for -1082G/A (p=0.01).

Of the 3,104 patients, 304 (9.8%) patients underwent TVR during follow-up. 51 (1.6%) patients died and 22 (0.7%) suffered from MI. After univariate analysis -2849AA, -1082AA and +4259GG genotypes of the IL-10 gene increased the risk for TVR significantly (p=0.005, p=0.03 and p= 0.001, respectively) (Table 2).

Table 2. Distributions of the polymorphisms, including the univariate analysis of investigated polymorphisms in association with TVR

Polymorphisms	Number of cases and controls geno-typed (N %)	Minor allele freq	Best fitting genetic model	TVR-rate for the different genotypes	P-value*
-2849G/A		0.28	Recessive		**0.005**
GG/GA	2,659 (92.5)			9.1%	
AA	215 (7.5)			14.9%	
-1082G/A		0.49	Recessive		**0.03**
GG/GA	2,040 (74.5)			8.7%	
AA	700 (25.5)			11.4%	
-592C/A		0.24	Additive		0.42
CC	1,693 (58.9)			10.0%	
CA	1,008 (35.1)			9.0%	
AA	172 (16.0)			9.3%	
+4259A/G		0.27	Recessive		**0.001**
AA/AG	2,643 (92.3)			9.1%	
GG	222 (7.7)			16.7%	

**P value determined by the Cox proportional regression model*

The effect of each polymorphism was adjusted for patient and intervention-re lated characteristics that were previously found to be related to TVR risk includ ing diabetes, hypertension, stenting, residual stenosis>20%, current smoking

and total occlusion, as well as age and gender. This analysis showed a significant association for the same three polymorphisms that were significantly associated with TVR in the univariate analysis. Furthermore, diabetes, stenting and total occlusion were significantly associated with TVR (Table 3a). Multivariable Cox regression analysis was performed, in which all four IL-10 polymorphisms were included. Subsequently, we performed multivariable Cox regression analysis in which we included all four IL-10 polymorphisms, and adjusted for the same clinical risk factors and selected in a backward stepwise manner the polymor phisms that were independently associated with TVR risk. Polymorphisms were removed from the model when their p-value was >0.10. A significant association was found for the IL-10 +4259GG genotype (p=0.001), implying that this poly morphism is an independent risk factor for TVR. Exclusion of the -1082G/A polymorphism, which was not in complete HW-equilibrium, did not alter the outcome.

Table 3a. Relative Risks (RR) of the univariate and multivariable analysis of the IL-10 polymorphisms in association with TVR for the total population (N=3,104)

	Raw RR (95%CI)	Adjusted for clinical variables RR (95%CI)	Multivariable analysis, including clinical variables and all 4 polymorphisms
Diabetes	_	1.5 (1.1-2.1)	1.5 (1.1-2.1)
Total occlusion	_	1.4 (1.0-1.9)	1.4 (1.01-1.9)
Hypertension	_	NS	NS
Stenting	_	0.7 (0.5-0.9)	0.7 (0.5-0.9)
Restenosis>20%	_	NS	NS
IL-10 -592C/A	0.9 (0.8-1.1)	NS	NS
IL-10 -2849G/A	1.7 (1.2-2.4)	1.7 (1.2-2.5)	NS
IL-10 -1082G/A	1.4(1.04-1.8)	1.4 (1.1-1.8)	NS
IL-10 +4259A/G	1.9 (1.4-2.7)	2.0 (1.4-2.8)	2.0 (1.4-2.9)

RR= relative risk, 95 CI= 95 confidence interval

Since we found a strong correlation between the IL-10 polymorphisms and stenting, we stratified patients to a stented and a non-stented population. The use of intracoronary stents was carried out at the discretion of the operator. The stented population consisted of 2,309 patients, of who 203 (8.8%) patients had to undergo a TVR. Both the stented population, and the non-stented population,

Table 3b. Relative Risks (RR) of the univariate and multivariable analysis of the IL-10 polymorphisms in association with TVR for the stented population (N=2,309)

	Raw RR (95%C.I)	Adjusted for clinical variables	Multivariable analysis, including clinical variables and all 4 polymorphisms
Diabetes	_	1.5 (1.1-2.1)	1.6 (1.1-2.4)
Total occlusion	_	1.4 (1.01-1.9)	NS
Hypertension	_	NS	NS
Restenosis>20%	_	NS	NS
IL-10 -592C/A	1.0 (0.8-1.3)	0.9 (0.7-1.1)	NS
IL-10 -2849G/A	1.2 (0.7-2.0)	1.7 (1.2-2.5)	NS
IL-10 -1082G/A	1.2 (0.9-1.7)	1.4 (1.1-1.8)	NS
IL-10 +4259A/G	1.5 (0.9-2.4)	2.0 (1.4-2.8)	1.6 (1.0-2.6)

RR= relative risk, 95 CI= 95 confidence interval

Table 3c. Relative Risks (RR) of the univariate and multivariable analysis of the IL-10 polymorphisms in association with TVR for the non-stented population (N=795)

	Raw RR (95%C.I)	Adjusted for clinical variables	Multivariable analysis, including clinical variables and all 4 polymorphisms
Diabetes	_	1.5 (1.1-2.1)	NS
Total occlusion	_	1.4 (1.01-1.9)	NS
Hypertension	_	NS	NS
Restenosis>20%	_	NS	NS
IL-10 -592C/A	0.8 (0.5-1.1)	0.9 (0.7-1.1)	NS
IL-10 -2849G/A	2.7 (1.6-4.7)	1.7 (1.2-2.5)	NS
IL-10 -1082G/A	1.6 (1.02-2.5)	1.4 (1.1-1.8)	NS
IL-10 +4259A/G	2.9 (1.7-5.0)	2.0 (1.4-2.8)	3.1 (1.8-5.4)

RR= relative risk, 95 CI= 95 confidence interval

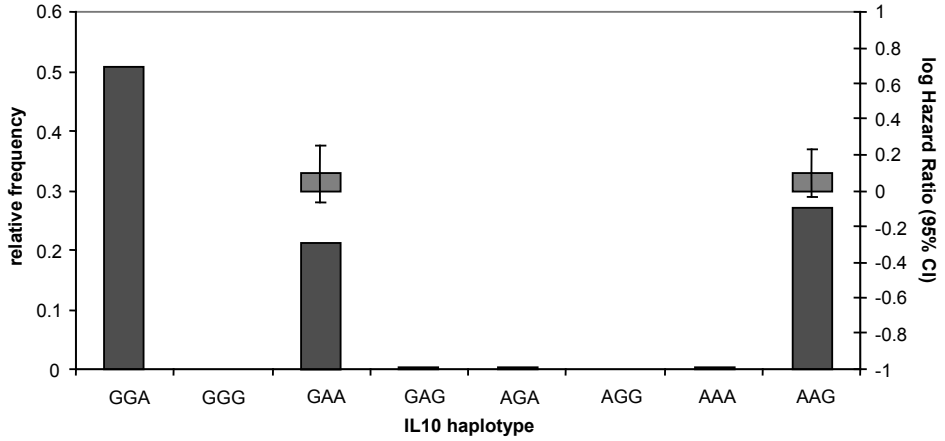
consisting of 795 patients, demonstrated a significant association for the same three polymorphisms as described earlier when adjusted for clinical variables. Multivariable analysis including all polymorphisms demonstrated the +4259GG genotype of the IL-10 gene to be associated with TVR. Results of this analysis are shown in tables 3b and 3c. Furthermore, they show that the effect of the +4259GG genotype of the IL-10 gene was more pronounced in the non-stented population.

A high level of linkage disequilibrium exists between the polymorphisms in the IL-10 gene. In order to determine whether these 3 polymorphisms are acting synergistically to confer risk to TVR, we performed two haplotype analyses. We first performed a combined analysis with -2849G/A, -1082G/A and +4259A/G. Of the 8 possible haplotypes, only 7 were observed with relative frequency > 0, and only 3 haplotypes had relative frequency > 1%, namely "GGA", "GAA", and "AAG" (Figure 1, red bars). The frequency of the GGA haplotype in patients with TVR was 0.47 compared to 0.51 for patients without TVR. For the GAA haplotype, the haplotype frequency was 0.30 for patients with TVR and 0.27 for patients without TVR. Furthermore, patients with TVR had a frequency of the AAG haplotype of 0.22 compared to 0.21 for patient without TVR. Compared to the wildtype haplotype ("GGA"), the log hazard ratios of the other haplotypes were 0.094, and 0.098, respectively (Figure1, blue bars). Differences were not statistically significant ($p>0.05$). Furthermore, since the -1082G/A polymorphism was not in Hardy-Weinberg equilibrium and the -2849G/A and the +4259A/G are in strong linkage disequilibrium ($D'=1$, $R^2=0.98$, pairwise LD as calculated by haploview) we performed separate haplotype analysis for the -2849G/A and the +4259A/G polymorphisms. The combination of these two polymorphisms did not give an additive effect (data not shown).

IL-10 is located in a cluster of IL-10 family genes on chromosome 1q32. Since the extent of linkage disequilibrium (LD) surrounding the IL-10 gene has not as yet been definitively characterized, we investigated whether the effect seen from IL-10 +4259GG could in fact be the result of LD with a neighboring gene. To this end, we genotyped polymorphisms in MAPKAPK2, IL-10, IL-19, IL-20 and IL-24 in a healthy population of unrelated Caucasian individuals (N=60). Haplo view was used to assess pairwise LD between polymorphisms of genes. Figure 2 reveals that the region spanning 217 kb is broken down into 4 blocks with a solid spine of LD. MAPKAPK2, IL-19, IL-20 and IL-24 seem to be independent of the IL-10 gene itself. IL-19, IL-20 and IL-24 form combined blocks indicating shared inheritance patterns of IL-20 with both IL-19 and

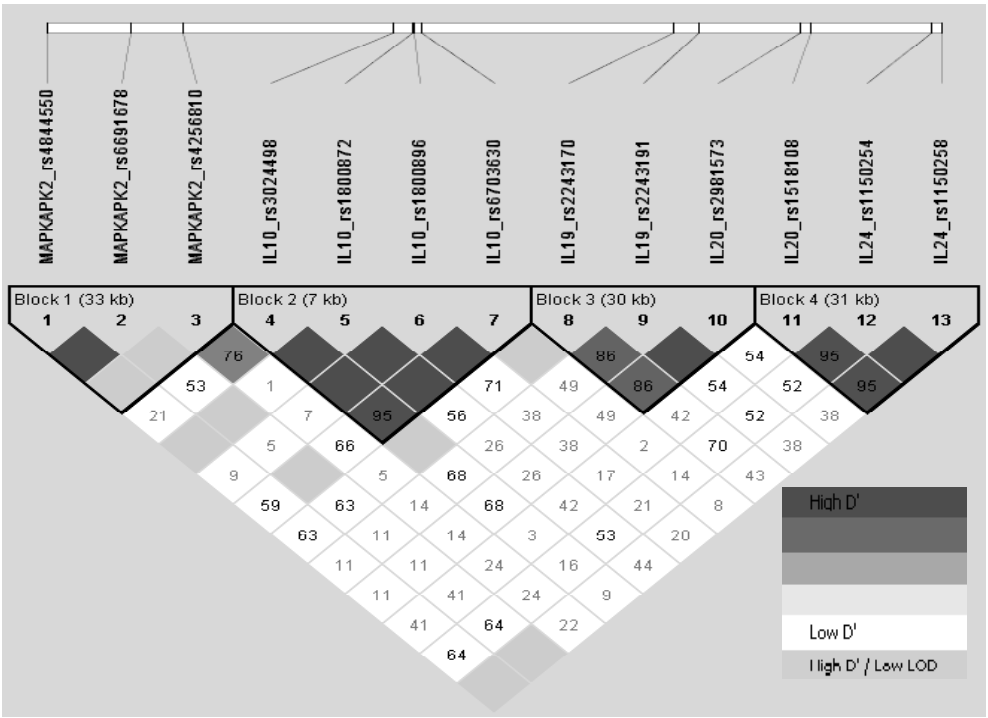
IL-24. This excludes the presence of *cis* effects of the IL-10 homologs or MAP KAPK2 on the IL-10 gene due to ancestral recombination. We therefore pro pose that IL-10 +4259 is the best predictor for risk to TVR.

Figure 1. IL-10 and haplotypes *For color figures see back of book.*



Haplotypes are presented in the following order of polymorphisms: 2849G/A, 1082G/A and the +4259A/G. Of the 8 possible haplotypes, only 3 haplotypes had relative frequency > 1 , presented by the red bars. The blue bars present log hazard ratios. Differences were not statistically significant p>0.05 .

Figure 2. Haplotype map of the IL-10 locus *For color figures see back of book.*



The haplotype map is made by Haploview version 3.2 software. The basis of this graphical representation is pairwise linkage disequilibrium LD between polymorphisms. D' values are shown in the boxes. In the event of D'=100 the boxes are empty. High D'/low LOD indicates no or low recombination between 2 polymorphisms. The region consists of 4 recombination blocks that encompass a 217 kb region between MAPKAPK2 and IL 24. Block 1 comprises the gene MAPKAPK2 , block 2 comprises the best known promoter polymorphisms of IL 10 and spans the whole IL 10 gene 592C/A rs1800872 , 2849G/A rs6703630 , 1082G/A rs1800896 and +4259A/G rs3024498 , block 3 shows a link be tween IL 19 and IL 20. Block 4 shows a link between the IL 20 and IL 24 genes.

Discussion

Inflammation is known to play a pivotal role in the development of restenosis af ter PCI.[4;5] Many inflammatory genes have already been investigated in relation to restenosis. However, the role of anti-inflammatory genes in restenosis is not fully understood. Therefore, we investigated in our large prospective GENDER study the effect of four different polymorphisms of the IL-10 gene in relation to

restenosis, defined by TVR in our study. After adjustment for clinical variables we found the -2849AA, the -1082AA and the +4259GG genotypes of the IL-10 gene to be highly associated with restenosis after PCI. After multivariable analy sis, in which we included all four polymorphisms and clinical factors previously associated with TVR, only the +4259GG genotype showed a significant associa tion. Possibly the +4259A/G polymorphism has an effect on restenosis by influ encing the mRNA stability.
Although these polymorphisms are highly linked, haplotype analysis did not pro vide any additional information with regards to risk of TVR. As the -1082G/A polymorphism was not in complete Hardy-Weinberg equilibrium, and as we can not be certain that this is due to selection of the population, since all patients have atherosclerosis or to genotype errors, we also performed multivariable analysis excluding this polymorphism. Exclusion of this polymorphism did not significantly alter the results. In this study, the novel IL-10 +4259A/G polymor phism conferred a significant risk to developing TVR, which is solely attributed to the IL-10 gene. Cis-acting variations in neighboring genes are unlikely to play a role since there are clear recombination points around the IL-10 gene, break ing the LD with neighboring genes.

Our assumption that a relationship exists between the IL-10 gene and the devel opment of restenosis after PCI was based on observations suggesting a critical role of this gene in the process of inflammation. Different cell types, includ ing human monocytes and T cells, produce IL-10. IL-10 inhibits the production of pro-inflammatory cytokines, including TNFα, probably by the induction of mechanisms inhibiting gene transcription and/or stability of mRNA. In human peripheral blood polymorphonuclear leukocytes, IL-10 interferes with the pro duction of various chemokines, including IL-8 necessary to sustain the recruit ment of different types of leukocytes for initiation or continued maintenance of an inflammatory process. Treatment of IL-1 activated human endothelial cells with IL-10 results in lower surface densities of intercellular adhesion molecule-1 and vascular-cell adhesion molecule-1 and reduced leukocyte adhesivity. In ad dition, IL-10 enhances the production of an IL-1 receptor antagonist that has anti-inflammatory activity directed against the effects of IL-1.[20]

Some studies found a relation between TNFα and IL-10 plasma levels.[21] Since we previously investigated the role of several polymorphisms in the TNFα gene[22], we analyzed whether those polymorphisms had an effect on our results. However, we did not find a significant association between the -238G/A and the -1031T/C polymorphisms of the TNFα gene and the four IL-10 polymorphisms

we examined in this study.

The functional effect of the polymorphisms we examined has been described previously. Koss et al. demonstrated that the A allele in the IL-10 promoter re gion at position -1082 was associated with decreased IL-10 production as mea sured by ELISA in lipopolysaccharide (LPS) stimulated whole blood in Crohn's disease patients and healthy controls (P=0.005, P=0.015, respectively).[13] Fur thermore, several studies have demonstrated that carriers of the -2849AA geno type have significantly lower IL-10 responsiveness upon stimulation with en dotoxin.[12;23] In some individuals allele G of the +4259 polymorphism produces less IL-10 transcripts as compared to the A allele(14), which points towards an allele-specific genetic regulation of protein levels of IL-10. Since we showed an increase in the risk of developing restenosis for the -1082AA, the -2849AA and the +4259GG genotypes, these data corroborate our hypothesis that lower levels of IL-10 may increase the risk of developing restenosis. However, the relevant stimulus and therefore the relevant transcription factor activating the IL-10 gene in the case of restenosis is largely unknown. Different transcription factors are known to either positively or negatively regulate the transcription of the IL10 gene.[24;25] The current functional data available on the regulation of the IL-10 gene is thus a mere indication of plausible scenarios that may or may not provide explanations in the case of restenosis.

Eefting et al. studied the involvement of IL-10 in neointima formation in a hyper cholesterolemic mouse model of cuff-induced stenosis of the femoral artery by IL-10 knocking-out or overexpression procedures. IL-10$^{+/-}$ mice were crossbred with ApoE*3-Leiden mice to generate hypercholesterolemic ApoE*3-Leiden x IL-10$^{-/-}$ mice. To achieve IL-10 overexpression in ApoE*3-Leiden mice, a single intramuscular injection of a murine IL-10 overexpression plasmid was performed followed by electroporation of the plasmid into the aorta. Knocking-out IL-10, in hypercholesterolemic ApoE*3-Leiden mice, resulted in a significant 1.9-fold increase of neointima surface as compared to ApoE*3-leiden IL-10$^{+/+}$ littermates (p=0.02). Conversely, a marked 45% inhibition on cuff-induced neointima forma tion was obtained after IL-10 overexpression (p=0.02). Electrodelivery of IL-10 vector leads to detectable IL-10 serum levels, with a sustained expression over the experimental period of 2-3 weeks. Finally, IL-10 overexpression stimulated endogenous IL-10 mRNA expression in the spleen and reduced the transcrip tional responses of several pro-inflammatory cytokines. They demonstrate a role of IL-10 in the development of neointima formation in hypercholesterolemic mice and the potential therapeutic effect of non-viral electrodelivery of IL-10

cDNA to inhibit post-angioplasty restenosis (data submitted).

Another study has examined the effect of recombinant human IL-10 (rhuIL-10) on intimal growth, after angioplasty or stent implantation, in hypercholesterol emic rabbits. [26] The main findings of their study were that systemic adminis tration of the anti-inflammatory cytokine rhuIL-10 successfully inhibits intimal hyperplasia after balloon injury or stent implantation in hypercholesterolemic rabbits. This protective effect is associated with a major inhibition of IL-1β re lease by circulating leukocytes and reduced infiltration of the arterial wall by ac tivated macrophages. RhuIL-10 has no apparent effect on lipid metabolism and no systemic toxicity in their animal model.[26] It is therefore possible that an IL-10 releasing stent may contribute to lowering the development of restenosis.

Study limitations

Circulating protein levels were not assessed in the present study. However, we believe that basal (pre-PCI) plasma levels of the gene product will not reflect the genetically determined differences in IL-10 increase after a trauma such as PCI. Moreover, local differences in response (in the vessel wall at the place of PCI) may not be reflected systemically. In humans it is impossible to measure gene products locally in the acute phase of treatment or the following days, and sev eral months later the causal trigger has probably already disappeared. Another potential limitation is that we examined TVR as our primary endpoint instead of angiographic outcomes, such as late loss. However, in clinical practice clinical re stenosis is an endpoint much more valuable than angiographic restenosis. Finally, as our study was conducted in a sample of Caucasian patients, extrapolation of the data to other ethnic groups should be done with great caution.

Conclusions

The present study shows that the -2849AA, the -1082AA and the +4259GG gen otypes of the gene coding for the anti-inflammatory cytokine IL-10 are a risk marker for the development of restenosis. Further investigation in other popula tions as well as the fine mapping of the IL-10 gene will provide further insight into the precise role of IL-10 in restenosis. Based on our findings screening pa tients for this genotype can lead to a better stratification of patients at increased risk for restenosis and thereby provide indications for improving individual tai lor-made treatment, since it may be a new target point for drug-eluting stents. The results of this study lend support to the broader hypothesis that genetic

programming of the inflammatory response plays a significant role in the devel opment of restenosis. Given the explorative nature of this analysis, our results need to be reproduced in other studies.

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