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Chapter 8

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

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Abstract

Genetic factors appear to be important in the process of restenosis after percutaneous coronary intervention (PCI), as well as in inflammation, a pivotal factor in restenosis. An important mediator in the inflammatory response is interleukin (IL)-10. Our aim was to study whether genetic variants in IL-10 predispose to the risk of restenosis. The GENetic DEterminants of Restenosis (GENDER) study included 3104 patients treated with successful PCI. Target vessel revascularization (TVR) was chosen as primary end point.

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 restenosis (-2849AA: relative risk (RR), 1.7, 95% confidence interval (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 anti-inflammatory genes also may be involved in developing restenosis. Furthermore, they may provide a new targeting gene for drug-eluting stents.

Introduction

An important limitation of the treatment of atherosclerotic lesions by percutaneous coronary intervention (PCI) is the occurrence of restenosis(1,2). Inflammatory 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 lymphocytes 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–macrophage colony-stimulation factor, inducible nitric oxide synthase and smooth muscle cell proliferation (8–10). Several of these factors have been demonstrated to be involved in the restenotic process (5,11). The interindividual difference among individuals in their ability to produce IL-10 appears to have a genetic origin (12–14). The heritability of the endotoxininduced IL-10 production has been estimated to be 74% in studies on monozygotic or dizygotic twins and nonrelated individuals (15). The gene encoding IL-10 contains variable sites (polymorphisms and microsatellite 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 (three in the promoter, one in the 3'UTR) in the IL-10 gene are related to the risk of developing restenosis after PCI.

Results

A total of 3146 patients had a complete follow-up (99.3%) with a median duration of 9.6 months (interquartile range 3.9 months). Out of 3146 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. Genotyping was successful in 2874 patients for the -2849G/A polymorphism, in 2740 patients for the -1082G/A polymorphism, in 2873 patients for the -592C/A polymorphism and in 2865 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 owing to the lack of DNA or inconclusive genotyping. Patients who could not be genotyped did not differ in any characteristic 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 ($P=0.01$).

Table 1 Demographic, clinical and lesion characteristics of 3104 patients with and without TVR

	Patients with TVR (n = *304)*	*Patients without TVR* (n = *2800)*	*Total* (n = *3104)*
Age (years)	61.7 ± 10.1	62.2 ± 10.8	62.1 ± 10.7
BMI (kg/m²)	26.9 ± 3.7	27.0 ± 3.9	27.0 ± 3.9
Male sex	220 (72.4%)	1996 (71.3%)	2216 (71.4%)
Diabetes	63 (20.7%)	390 (13.9%)	453 (14.6%)
Hypercholesterolemia	188 (61.8%)	1702 (60.8%)	1890 (60.9%)
Hypertension	138 (45.4%)	1121 (40.0%)	1259 (40.6%)
Current smoker	62 (20.4%)	700 (25.0%)	762 (24.5%)
Family history of MI	121 (39.8%)	977 (34.9%)	1098 (35.4%)
Previous MI	109 (35.9%)	1130 (40.4%)	1239 (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%)	1881 (67.2%)	2079 (67.0%)
Multivessel disease	148 (48.7%)	1284 (45.9%)	1432 (46.1%)
Peripheral vessel disease	12 (3.9%)	92 (3.3%)	104 (3.4%)
Lipid-lowering medication	171 (56.3%)	1516 (54.1%)	1687 (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)

Abbreviations: BMI, body mass index; CABG, coronary artery bypass grafting; LAD, left anterior descending branch of the left coronary artery; MI, myocardial infarction; PCI, percutaneous coronary intervention; RCX, circumflex branch of the left coronary artery; VR, target vessel revascularization. Age is mean ±s.d.; other variables are percentage of patients.

Of the 3104 patients, 304 (9.8%) patients underwent target vessel revascularization (TVR) during follow-up. Fifty-one (1.6%) patients died and 22 (0.7%) suffered from myocardial infarction (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).

The effect of each polymorphism was adjusted for patient and intervention-related characteristics that were previously found to be related to TVR risk including 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 polymorphisms that were independently associated with TVR risk. Polymorphisms were removed from the model when

their P-value was 40.10. A significant association was found for the IL-10 +4259GG genotype (P=0.001), implying that this polymorphism 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.

As 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 2309 patients, of who 203 (8.8%) patients had to undergo a TVR. Both the stented population and the non-stented population, 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 Table 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.

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

Polymorphisms	*Number of cases and controls genotyped (N%)*	*Minor allele frequency*	*Best-fitting genetic model*	*TVR rate for the different genotypes (%)*	*P-value**
−2849G/A		0.28	Recessive		0.005
GG/GA	2659 (92.5)			9.1	
AA	215 (7.5)			14.9	
−1082G/A		0.49	Recessive		0.03
GG/GA	2040 (74.5)			8.7	
AA	700 (25.5)			11.4	
−592C/A		0.24	Additive		0.42
CC	1693 (58.9)			10.0	
CA	1008 (35.1)			9.0	
AA	172 (16.0)			9.3	
+4259A/G		0.27	Recessive		0.001
AA/AG	2643 (92.3)			9.1	
GG	222 (7.7)			16.7	

Abbreviation: TVR, target vessel revascularization.
*P-value determined by the Cox proportional regression model.

Table 3 Relative risks (RR) of the univariate and multivariable analysis of the IL-10 polymorphisms in association with TVR for the
(a) total population (N = 3104); (b) stented population (N = 2309) and (c) non-stented population (N = 795)

	Raw RR (95% CI)	*Adjusted for clinical variables RR (95% CI)*	*Multivariable analysis, including clinical variables and all four polymorphisms*
(a)			
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)
(b)			
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)
(c)			
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)

Abbreviations: 95% CI = 95% confidence interval; IL, interleukin; NS, not significant; RR = relative risk; TVR, target vessel revascularization.

A high level of linkage disequilibrium exists between the polymorphisms in the IL-10 gene. In order to determine whether the three significant associated polymorphisms with TVR are acting synergistically to confer risk to TVR, we performed two haplotype analyses. We did not include the -592C/A polymorphism in the haplotype analysis, as it did not show a significant association with TVR. Furthermore, the R^2 value for this polymorphism is much lower (pairwise linkage disequilibrium (LD) between -592 and +4259 has R^2=0.12).

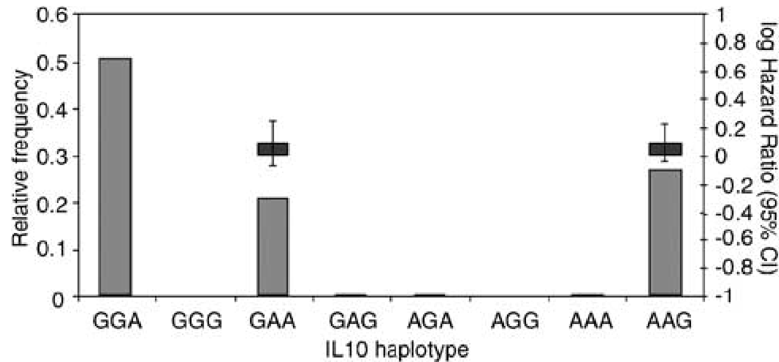

Figure 1 IL-10 and haplotypes. Haplotypes are presented in the following order of polymorphisms: -2849G/A, -1082G/A and +4259A/G. Of the eight possible haplotypes, only three haplotypes had relative frequency >1%, presented by the red bars. The blue bars present log hazard ratios. Differences were not statistically significant (P>0.05).

We first performed a combined analysis with -2849G/A, -1082G/A and +4259A/G. Of the eight possible haplotypes, only seven were observed with relative frequency >0, and only three 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 0.22 for patients with TVR and 0.21 for patients without TVR. Furthermore, patients with TVR had a frequency of the AAG haplotype of 0.30 compared to 0.27 for patient without TVR. Compared to the wild-type haplotype ('GGA'), the log hazard ratios of the other haplotypes were 0.094 and 0.098, respectively (Figure 1, blue bars). Differences were not statistically significant (P>0.05). Furthermore, as the -1082G/A polymorphism was not in HW equilibrium and the-2849G/A and the +4259A/G are in strong linkage disequilibrium (D' = 0.96, R^2=0.92 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. As the extent of LD surrounding the IL-10 gene has not as yet been definitively characterized, we investigated whether the effect seen from the examined IL-10 polymorphisms could in fact be the result of LD with a neighboring gene. To this end, we genotyped polymorphisms in mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), IL-10, IL-19, IL-20 and IL-24 in a healthy population of unrelated Caucasian individuals (*N*=60). Haploview was used to assess

pairwise LD between polymorphisms of genes. Figure 2 reveals that the region spanning 217 kb is broken down into four 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. These data are in line with the haplotype blocks as defined by HAPMAP for a Caucasian population of European descent. In our study, this finding excludes the presence of *cis* effects of the IL-10 homologs or MAPKAPK2 on the IL-10 gene owing to ancestral recombination and reveals that the association of the IL-10 gene with restenosis is independent of flanking genes.

Figure 2 Haplotype map of the IL-10 locus. 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. The region consists of four 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

Discussion

Inflammation is known to play a pivotal role in the development of restenosis after 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. Different cell types, including human monocytes and T cells, produce IL-10. IL-10 inhibits the production of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNFα) (16). Furthermore, it interferes with the production of various chemokines (17). 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 (18). In addition, IL-10 enhances the production of an IL-1 receptor antagonist that has anti-inflammatory activity directed against the effects of IL-1 (19).

After adjustment for clinical variables, we found the -2849AA, -1082AA and +4259GG genotypes of the IL-10 gene to be highly associated with restenosis after PCI. After inclusion of all four polymorphisms and clinical factors previously associated with TVR, only the +4259GG genotype showed a significant association. Possibly, the +4259A/G polymorphism has an effect on restenosis by influencing the mRNA stability.

Haplotype analysis did not provide any additional information with regards to risk of TVR. As the -1082G/A polymorphism was not in complete HW equilibrium, and as we cannot be certain that this is due to selection of the population, or to genotype errors, we also performed multivariable analysis excluding this polymorphism. Exclusion of this polymorphism did not significantly alter the results. With the current chosen polymorphisms, we are able to characterize about 70% of the four common haplotypes in the IL-10 gene. However, we cannot exclude the possibility that less common haplotypes, characterized by further tagging polymorphisms (as provided by the HAPMAP project, www.hapmap.org), could provide more information with regards to the risk of restenosis.

The results of our study reveal that association of the IL-10 gene with restenosis is independent of flanking genes, as *cis*-acting variations in neighboring genes are unlikely to play a role given that there are clear recombination points around the IL-10 gene, breaking the LD with neighboring genes. These findings are in line with the results of the HAPMAP project in a Caucasian population of European descent.

Some studies found a relation between TNFα and IL-10 plasma levels (20). As we previously investigated the role of several polymorphisms in the TNFα gene (11) 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 region at position -1082 was associated with decreased IL-10 production as measured by enzyme-linked immunosorbent assay (ELISA) in lipopolysaccharide (LPS)-stimulated whole blood in Crohn's disease patients and healthy controls (P=0.005, P=0.015, respectively) (13). Furthermore, several studies have demonstrated that carriers of the -2849AA genotype have significantly lower IL-10 responsiveness upon stimulation with endotoxin (12,21). 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. As we showed an increase in the risk of developing restenosis for the -1082AA, -2849AA and +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 IL-10 gene (22, 23). 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 hypercholesterolemic mouse model of cuff-induced stenosis of the femoral artery by IL-10 knocking-out or overexpression procedures. 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 formation was obtained after IL-10 overexpression (P=0.02) (24).

Another study has examined the effect of recombinant human IL-10 (rhuIL-10) on intimal growth, after angioplasty or stent implantation, in hypercholesterolemic rabbits (25). The main findings of their study were that systemic administration 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-1b release by circulating leukocytes and reduced infiltration of the arterial wall by activated macrophages. RhuIL-10 has no apparent effect on lipid metabolism and no systemic toxicity in their animal model (25). 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) lasma 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 several months later the causal trigger has probably already disappeared.

Furthermore, the lack of data on the effect of the presently used drug-eluting stents on TVR and its relationship with the IL-10 polymorphisms is a limitation of our study. Another potential limitation is that we examined TVR as our primary end point instead of angiographic outcomes, such as late loss. However, in clinical practice, clinical restenosis is an end point 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, -1082AA and +4259GG genotypes of the gene coding for the anti-inflammatory cytokine IL-10 are a risk marker for the development of restenosis. Further investigation in other populations 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 patients for this genotype can lead to a better stratification of patients at increased risk for restenosis and thereby provide indications for improving individual tailor-made treatment, as 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 development of restenosis. Given the explorative nature of this analysis, our results need to be reproduced in other studies.

Materials & Methods

GENDER project

Study design

The present study population has been described previously (26). In brief, the 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 MI were excluded. Also excluded from analysis were patients suffering from events occurring within 1 month after PCI, as 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 300mg and heparin 7500 IU. The use of intracoronary stents and additional medication, such as glycoprotein IIb/IIIa inhibitors, were carried out at discretion of the operator. In case a stent was implanted, patients received either ticlopidin or clopidogrel for at least 1 month following the procedure depending on local practice. During the study, no drug-eluting stents were used.

Follow-up and study end points

Follow-up lasted for at least 9 months, except when a coronary event occurred. Patients were either seen in outpatient clinics or contacted by telephone. TVR, either by PCI or coronary artery bypass grafting (CABG), was designated the primary end point, as 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 before the PCI procedure.

Genetic methodology

Blood was collected in ethylene diaminetetraacetate (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 polymorphisms were selected from literature, and databases on the web, criteria used were frequency of the rare allele and a possible functional effect (8,10,13,14). To assay these polymorphisms, we used a MassArray platform according to manufacturer's protocols. Two multiplex assays were designed using Assay designer software (Sequenom, Hamburg, Germany). After polymerase chain reaction (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 Autoflex (Bruker, Wormer, Netherlands) matrix-assisted laser desorption\ionization 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. Positive and negative controls uniquely distributed in each 384 wells plate were also consistent. Cluster plots were made of the signals from the low and the high mass allele. Two independent researchers carried out scoring. Disagreements or vaguely positioned dots produced by Genotyper 3.0 (Sequenom) as well as all wells that had 50% or more failed SNPs were excluded from analysis.

Statistical analysis

Deviations of the genotype distribution from that expected for a population in HW equilibrium was tested using the χ^2 test with one degree of freedom. Allele frequencies were determined by gene counting, the 95% CIs of the allele frequencies were calculated from sample allele frequencies, based on the approximation of the binominal and normal distributions in large sample sizes.

Continuousvariables are expressed as mean± standard deviation and were compared by means of the unpaired, two-sided *t*-test. Discrete variables are expressed as counts or percentages and were compared with the χ^2. In the first stage, the association between the IL-10 polymorphisms and TVR was assessed using the Cox proportional regression model under a co-dominant genetic 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 (27). Multivariable regression analysis of the TVR risk was performed with all IL-10 polymorphisms, using a stepwise backward selection algorithm. 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* (28). Evaluation 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. Haplotype blocks were assigned under the algorithm of solid spine of LD as provided by the software. 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.

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