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Associatiestudies van cytokine- en stress eiwit genpolymorfismen in hartfalen en transplantatie gerelateerde complicaties

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Cécile Holweg

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	Dr. R.A. de Weger
Copromotor:	Dr. C.C. Baan

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Aan Pa en Ma

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Cécile Holweg
Willem Weimar
André Uitterlinden
Carla Baan

Based on part of review:
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Introduction

Heart transplantation is an effective treatment for patients suffering from end-stage heart failure. Most patients who are referred to cardiac transplantation suffer from heart failure due to dilated cardiomyopathy (CMP), ischemic heart disease (IHD), valvular heart disease or congenital malformations. Over the last decades, the success of heart transplantation increased largely due to the introduction of new, more effective and more specific immunosuppressive drugs. However, despite these improvements, severe complications still remain, including acute rejection (AR), infections, graft vascular disease (GVD), also called transplant coronary artery disease (TCAD), renal failure and post transplant lymphoproliferative disorders (PTLD). These complications significantly affect the well being and survival of cardiac allograft recipients.

Acute rejection is an inflammatory process that is characterized by infiltration of mononuclear cells leading to myocyte damage or even necrosis. Acute rejection after heart transplantation is diagnosed by histological examination of routinely taken endomyocardial biopsies. It usually occurs within the first weeks to months after transplantation, but may even been encountered after years. Nowadays, acute rejection can in general adequately be treated and is rarely fatal. However, acute rejection and its treatment are associated with development of infections (CMV), GVD and PTLD after heart transplantation. Therefore, preventing acute rejection is advantageous for short and long term complications. GVD, is a complication that influences the long-term survival. About 30% of the cardiac recipients dies from this complication.[1] GVD is characterized by progressive and diffuse concentric fibrosis and smooth muscle cell proliferation with collagen accumulation leading to intimal thickening of the epicardial as well as the intramyocardial arteries.[2] The disease is irreversible, and to date, there is no treatment. Other frequently seen complications are infections, renal insufficiency and PTLD. These complications are largely attributed to the immunosuppressive medications.

All these complications after transplantation originate from a combination of allogen-dependent and -independent factors in the context of recipient and donor variability's under circumstances of immunosuppression. A number of these factors are genetically controlled by variations in genome sequences, polymorphisms, that consequently might influence the development and progression of transplant related problems. Of the more than two million known polymorphisms in the human genome, in transplantation medicine specific attention has been paid to variations in the genes encoding for immune mediators like cytokines. These

soluble mediators play a critical role in the pathogenesis of heart failure and of the complications observed after organ transplantation.

Cytokines

Cytokines are small soluble polypeptides that mediate immune responses, inflammatory reactions and control cellular interactions. The family of cytokines includes the interleukins (e.g., IL-2, IL-4, IL-10), interferons (e.g., IFN-γ), lymphokines (e.g., TNF-α, TNF-ß), growth factors (e.g., TGF-ß, VEGF, bFGF, PDGF-α and GM-CSF), and chemokines (e.g., MCP-1, MIP-1α, MIP-1ß).[3] Most cytokines are produced by a variety of cell types. They exert their functions by binding to specific cell bound receptors on target cells, which then transmit the extracellular binding event into an intracellular signal.[3] Cytokines influence synthesis and actions of other mediators of the cytokine family, leading to a cytokine cascade that, in turn, induces, enhances or inhibits expression of a number of cytokine genes.[4] Cytokines regulate the activation, proliferation, differentiation, acquisition, but also death of immune cells. Most cytokines perform their functions close to the place where they are secreted. They can act on the cell by which they are produced (autocrine) or on a nearby cell (paracrine). However, they can also act at a distance (endocrine), when they are produced in large amounts and released into the circulation.[5] Most cytokines are able to act on multiple target cells (pleiotropy), and overlap in function (redundancy), which is necessary for an adequate immune or inflammatory response.[3] Besides to the cell surface receptor, many cytokines bind to soluble receptors, which are sometimes released ligand binding domains of the actual surface receptor.[3]

Role of cytokines in heart failure

Cytokines are associated with heart failure that has been proposed to be a state of immune activation.[6] Numerous studies have demonstrated elevated circulating levels and heightened expression of cytokines in the diseased myocardium. A correlation was found between TNF-α and IL-6 circulating levels and myocardial expression and deteriorating New York Heart Association (NYHA) class.[4, 6] TNF-α stimulates extracellular matrix protein production that induces ventricular remodeling. In addition, it produces reactive oxygen species, thereby causing endothelial dysfunction and stimulating myocyte hypertrophy. [7] In addition, TNF-α can induce apoptosis in a wide variety of cells.[5] Another cytokine, IL-1 has been shown to depress myocardial contractility, it promotes the interaction with

circulating leukocytes and induces activation of macrophages and proliferation of monocytes.[6, 8] IL-6 is thought to be involved in regulating cardiomyocyte hypertrophy and apoptosis.[9] In relation to the raised levels of pro-inflammatory cytokines, decreased levels of anti-inflammatory mediators, like TGF-ß and IL-10 were measured in patients with congestive heart failure.[10] Apart from its anti-inflammatory properties, TGF-ß also has pro-fibrotic properties as it regulates extracellular matrix production. These latter properties of TGF-ß might play a role in the pathogenesis of end-stage cardiac failure.[11]

Cytokines in transplantation

Cytokines play an important role in regulating inflammatory responses, induced by allogen independent and dependent triggers. Various peri-operative events, like brain death of the donor, ischemia and reperfusion, induce tissue injury, e.g., endothelial damage. Under physiological circumstances, the healthy endothelium is able to reduce oxidative stress and to inhibit thrombus formation, leukocyte adhesion and smooth muscle cell proliferation. Injury however, activates the endothelium resulting in an altered endothelial cell function and a subsequent inflammatory response with subsequent smooth muscle cell proliferation and matrix deposition.[12] In a response to this damage, the immune system is activated and cytokines, like TNF-α, IL-1ß, IL-6, IFN-γ, MCP-1, and TGF-ß are secreted by these activated endothelial cells.[13, 14] This leads to upregulation of the major histocompatibility antigens (HLA) and adhesion molecules on endothelial cells, subsequently promoting recruitment of host leukocytes to the graft.[14] This allogen-independent tissue injury leads to a higher immunogenicity of the transplanted organ, subsequently resulting in a more pronounced allogen-dependent processes, i.e., acute rejection. In acute rejection, T-cells, macrophages, natural killer cells and B-cells infiltrate the graft and produce various cytokines. Higher intragraft cytokine mRNA and protein expression, but also circulating levels of, among others, IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, and TGF-ß, were reported during acute rejection episodes.[13, 15] IFN-γ secreted by damaged endothelial cells recruits leukocytes into the graft and activates them.[16] IFN-γ is also an activator of vascular endothelial cells and potentiates many of the action of TNF-α on endothelial cells.[5] In addition, TNF-α and IFN-γ are able to upregulate MHC class I and II, respectively, thereby enhancing the immunogenicity of the graft. These cytokines can upregulate cytotoxicity of infiltrated T-cells (IFN-γ) or can have direct cytotoxic effects (TNF-α).[14] IL-2 promotes differentiation and clonal expansion of activated T-lymphocytes. Furthermore, IL-2 supports B-cell growth, induces the secretion of antibodies by B-cells and augments the cytolytic activity of NK-cells.[17]

However, on the other hand, IL-2 plays a role in priming T-cells to undergo apoptotic cell death, which serves as a feedback regulator of clonal expansion stimulates the clonal expansion of T-cells.[18] IL-6 can induce chemokine production and leukocyte recruitment.[15] While IL-10, IL-4 and TGF-ß may suppress the inflammatory response, and abrogate the production of inflammatory cytokines.[16]
GVD is another complication after transplantation in which cytokines play a role. It is a chronic inflammatory process and triggers for this inflammatory response can both be both allogen-independent events and donor specific immune responses. The development of GVD, is the result of an ongoing immune reactivity, whereby many cytokines and growth factors are involved.[19] The literature tells us that among others, TNF-α, IL-1, IFN-γ, IL-2, IL-6, TGF-ß, PDGF and bFGF, but also chemokines like MCP-1 are implicated.[13, 19, 20] A well studied mediator in GVD is TGF-ß, a multifunctional growth factor that plays a role in wound healing. It is involved in cell differentiation and controls the extracellular matrix production and degradation.[19] TNF-α, TGF-ß and PDGF-α regulate smooth muscle cell proliferation. In addition, PDGF and TNF-α play a role the local attraction of leukocytes and enhance collagen synthesis.[19, 20] bFGF is a mitogen for endothelial cells and stimulates their migration.[20] IL-1, IL-2, IL-6 and PDGF-α are able to promote creation of connective tissue matrix and migration of vascular smooth muscle cells.[21] Thus cytokines play a role in the chronic inflammatory process, however, the mechanism of the development of GVD remains to be investigated.

Cytokines in renal failure

Renal insufficiency in the end leading to dialysis after heart transplantation is a frequently seen complication resulting from the use of nephrotoxic immunosuppressive drugs, the calcineurin inhibitors (Cyclosporin and Tacrolimus). At 4 years after heart transplantation, 24% of the recipients had an impaired renal function.[22] Renal failure is characterized by interstitial fibrosis, tubular atrophy, glomerular vascular lesions and accumulation of extracellular matrix. [23] In the development of renal failure, the most extensive studied cytokine is TGF-ß. It has been shown that Cyclosporin increases TGF-ß expression, a correlation exists between TGF-ß expression in the kidney and renal function.[24, 25] The properties of TGF-ß, like regulation of extracellular matrix formation and the other profibrotic properties contribute to the development of renal insufficiency.

Heme Oxygenase-1

Tissue injury induced by oxidative stress is implicated in the pathogenesis of inflammatory diseases such as atherosclerosis and myocardial ischemia.[26, 27]

Oxidative stress is also an important trigger for the development of complications after heart transplantation, like acute rejection (AR) and transplant coronary artery disease (TCAD).[2, 28] The cytoprotective enzyme Heme Oxygenase (HO)-1 is found to be expressed by several cells and tissues in response to oxidative injury. HO-1 catalyzes the degradation of heme into biliverdin (subsequently converted to bilirubin), iron (next converted into ferritin) and carbon monoxide (CO). The end products of this reaction, bilirubin, CO and ferritin, all have anti-oxidant functions and are thought to be responsible for the protective effects of HO-1, including anti-oxidant, anti-inflammatory, anti-apoptotic, anti-proliferative and vasodilatory effects.[26]

Gene polymorphisms

A polymorphism is a location of the genome that varies in sequence between individuals and is present in a considerable number of individuals in a population, traditionally at least 1%.[29] Each common variant of a polymorphic locus is called an allele. An individual may carry two different alleles, as they inherit one of each parent. The combination of these two alleles is called a genotype. Polymorphisms can be either anonymous or functional. An anonymous polymorphism has no known effect on transcription, translation or function of a protein. In contrast, functional polymorphisms do have biological consequences. Polymorphisms can also be in linkage disequilibrium with another truly functional polymorphism at a disease locus, in which case the actually genotyped polymorphism then acts as a marker.[30]
There are different kinds of gene polymorphisms. Most common is the single nucleotide polymorphism (SNP), which a change of a single basepair in the genomic DNA and occur, on average, every 400 bases.[31, 32] SNP's can have an effect on gene function. For example, a SNP located in a promoter region might influence the amount of mRNA produced. A SNP in a coding sequence can alter the resultant amino acid and thereby composition and thus the function of a protein, such as the binding affinity to the receptor or enzymatic activity. An example of a SNP in a cytokine gene is the transition form A to G at position -308 in the promoter of the TNF-α gene.[33]

Another class of polymorphisms is the simple sequence repeats. These simple sequence repeats or variable number of tandem repeats (VNTR) are also called microsatellites, when the repeat units have 1-6 basepairs, or minisatellites when the repeat unit contains more nucleotides.[34] Most common are the dinucleotide and trinucleotide repeats. VNTR's might also influence the function of a gene, but

more likely they are in linkage with a functional polymorphism elsewhere in the gene. The $(CA)_n$ repeat in the first intron of the IFN-γ gene is an example of a VNTR.[35]

A third category of gene polymorphisms involves insertions or deletions. They can be as small as one base, in which case they can also be classified in the category of SNP's. But they can also exist of a few bases, one ore more exons or even a whole gene. An example of an insertion/deletion polymorphisms is the C at position +72 in the TGF-ß1 gene.[36]

In clinical terms, gene polymorphisms can influence the cause of heart failure and can cause differences in susceptibility to complications after transplantation, time to onset or the severity of a complication, but also in the way a recipient responds to drug therapy or immunosuppressive therapy or to treatment of transplant related problems. Studying frequencies and patterns of polymorphisms, in genes known to be involved in heart failure or transplant related complications, in recipients, organ donors and controls, can give us information about processes in the development and progression of these disorders. From these studies we can in theory identify risk factors for the development of these disorders and this might ultimately allow us to use this information in the clinical practice.

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Chapter 2

Aim of the thesis

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The aim of this thesis is to identify genetic factors involved in the pathogenesis of heart failure and of the frequently occurring complications after heart transplantation.

Cytokines and oxidative stress proteins are associated with the pathogenesis of heart failure, i.e., ischemic heart disease (IHD) and dilated cardiomyopathy (CMP) and in complications after heart transplantation, i.e., acute rejection (AR), graft vascular disease (GVD), also called transplant coronary artery disease (TCAD), and renal failure. Gene polymorphisms can have biological consequences and might therefore influence the susceptibility to cardiovascular diseases and complications after heart transplantation. Therefore, we focussed on genetic variations, polymorphisms, in the cytokine genes IL-2, IFN-γ and TGF-ß1 and the oxidative stress molecule HO-1, in an attempt to find associations with the clinical problems of heart transplant recipients.

Two important cytokines involved in the development of AR and in the pathogenesis of GVD are the cytokines IL-2 and IFN-γ. **Chapter 3** describes an association study of a dinucleotide repeat $(CA)_m(CT)_n$ polymorphism in the 3'flanking region of the IL-2 gene in relation to AR after heart transplantation. In **chapter 4**, the results of a study of another polymorphism in the IL-2 gene, a T/G transition at position -330 in the promoter region, and of a repeat polymorphisms $(CA)_n$ in the first intron of the IFN-γ gene in relation to both AR and GVD are reported. Both polymorphisms described in this chapter are associated with the production capacity for these cytokines.

In the following three chapters, two polymorphisms in the TGF-ß gene are studied in heart failure, **chapter 5**, in the development of GVD, **chapter 6**, and in relation to renal failure after heart transplantation, **chapter 7**. TGF-ß1 is a multifunctional cytokine that is thought to contribute to all three disorders. The polymorphisms in the TGF-ß1 gene are single nucleotide polymorphisms, +869 T/C and +915 G/C, which alter amino acids in the leader sequence, codon 10 Leu/Pro and codon 25 Arg/Pro, respectively. These amino acid substitutions might have consequences for the production of the TGF-ß1 protein and thus can influence the susceptibility to the above mentioned causes of heart failure leading to transplantation and to transplant related complications.

Recently, it became clear that stress proteins, like Heme Oxygenase (HO)-1, might play a role in diseases in which oxidative stress is implicated. As oxidative stress may be involved in heart failure and transplant related complications like AR and TCAD, it can be suggested that HO-1 is involved in heart failure and complications

after transplantation. The HO-1 promoter contains a repeat polymorphism $(GT)_n$ that controls the enzymatic activity of HO-1. In **chapter 8** the role of HO-1 in the development and progression of transplant coronary artery disease was investigated. We studied intragraft mRNA and protein expression in the first year after transplantation. Furthermore, we investigated a repeat polymorphisms $(GT)_n$ in the promoter region of the HO-1 gene, in an attempt to identify predisposed patients for TCAD.

In **chapter 9** we describe the results from a study in which we attempted to find an association between the repeat polymorphism $(GT)_n$ in the HO-1 gene and the original diseases of the recipients leading to transplantation and to both AR and TCAD after transplantation.

In **chapter 10**, we give an overview of the literature, including our own results, and discuss the results of these studies. We wanted to ascertain whether these studies together have resulted in the identification of genetic risk factors and whether we already are able to use these potential risk factors in the clinical practice. In other words, can we already give practical guidelines for the monitoring and treatment of our patients, with the results of genetic association studies obtained so far.

And finally, **chapter 11** gives a summary of the studies described in this thesis and the conclusion drawn from these studies.

Chapter 3

The effect of HLA-DR matching on acute rejection after clinical heart transplantation might be influenced by an IL-2 gene polymorphism

Cécile Holweg
Annemiek Peeters
Aggie Balk
André Uitterlinden
Bert Niesters
Lex Maat
Willem Weimar
Carla Baan

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Abstract

To examine whether genetic factors are involved in the development of acute rejection (AR), we investigated a $(CA)_m(CT)_n$ repeat in the 3' flanking region of the IL-2 gene. We genotyped 290 heart transplant recipients with and without AR (ISHLT criteria ≥3A) and 101 controls.

The frequency of allele 135 of the repeat and its genotype distribution (carriers/non carriers) were significantly associated with freedom from AR ($p=0.03$ and $p=0.02$ respectively). We also found interaction between allele 135 and HLA-DR matching. More carriers of allele 135 with no or 1 mismatch remained free from AR compared to patients without the allele ($p=0.01$). This was not found in the HLA-DR group with 2 mismatches.

HLA-DR matching might only be effective in reducing acute rejection after heart transplantation in recipients who carry allele 135 of the $(CA)_m(CT)_n$ repeat in the 3' flanking region of the IL-2 gene.

Introduction

After clinical heart transplantation, acute rejection (AR) is a common and severe complication that develops under the influence of cytokines. A dominant cytokine involved in the pathway leading to rejection is interleukin (IL)-2. IL-2 promotes differentiation and clonal expansion of activated T-lymphocytes. Furthermore, IL-2 supports B-cell growth, induces the secretion of antibodies by B-cells, and augments the cytolytic activity of natural killer cells. However, IL-2 also plays a role in priming T-cells to undergo apoptotic cell death, which serves as a feedback regulator of clonal expansion.[1]

To prevent allograft rejection, patients receive immunosuppressive agents, of which some are particularly directed against the production of IL-2 or against the consequences of IL-2.[2] However, using the same standardized immunosuppressive medication, only a proportion of patients will reject their grafts, while others remain free from rejection. Alloreactivity is largely dependent on genetically determined differences in HLA (chromosomal location: 6p21) between donor and recipient. However, the strength of the anti-donor response, eventually resulting in graft damage, might also be genetically determined, e.g., by variations in cytokine genes.

Given the prominent role of IL-2 in the rejection process, it can be hypothesized that polymorphisms in the IL-2 gene (chromosomal location: 4q26-q27) are associated with rejection. Several polymorphisms in the IL-2 gene have been described: a single nucleotide polymorphism (SNP) in the promoter (-330, T→G), a silent SNP in the leader sequence (+166, G→T), and a dinucleotide repeat polymorphism $(CA)_m(CT)_n$ in the 3' flanking region of the IL-2 gene.[3, 4] One of these polymorphisms, the $(CA)_m(CT)_n$ repeat, has previously been implicated in a human inflammatory disease, i.e., ulcerative colitis.[5] To date, this $(CA)_m(CT)_n$ repeat has not been analyzed in relation to AR after organ transplantation.

We investigated whether a specific $(CA)_m(CT)_n$ repeat in the 3' flanking region of the IL-2 gene is associated with the development of AR after clinical heart transplantation, and if so, whether there would be interaction with other known risk factors for AR, e.g., the number of HLA mismatches with the donor.

Materials and methods

Patients and controls

We studied the IL-2 $(CA)_m(CT)_n$ repeat in 290 out of 329 heart transplant recipients, who received transplants between 1984 and 1998 in Rotterdam, while 101 healthy volunteers served as controls for genotype frequencies. All patients received cyclosporin A and low-dose steroids as maintenance immunosuppressive therapy. Both the patient and control population consisted mainly (279/290 and 96/101 respectively) of Caucasians. For analyses of the IL-2 $(CA)_m(CT)_n$ repeat, the patient group was divided into patients with (n=223) and without (n=67) AR episodes in the first year after transplantation. AR was histologically diagnosed in endomyocardial biopsies according to the criteria of the International Society for Heart and Lung Transplantation (ISHLT, ≥3A). There were no significant differences between patients with or without AR for recipient age (mean 49.5±9.6 and 48.1±15.1; p=0.19, Students t-test), recipient gender distribution (M/F: 188/35 and 57/10; p=0.88, χ^2-test), underlying disease (ischemic heart disease/dilated cardiomyopathy/other: 122/88/13 and 36/30/1; p=0.30, χ^2-test), cold ischemic time (mean 165±38 and 173±41; p=0.16, Students *t* test), induction therapy (no induction therapy/OKT3/ATG/BT563: 54/70/62/37 and 20/25/16/6; p=0.32, χ^2-test) and the mean number of HLA A, B mismatches (mean 3.0±0.9 and 2.9±0.9, p=0.58, Students *t* test). However, the mean number of HLA-DR mismatches was different between patients with and without AR (mean n=1.5±0.6 and n=1.3±0.7; p=0.03, Students *t* test).

DNA isolation and genotyping

Genomic DNA for genotyping was isolated from 10^6 peripheral blood mononuclear cells (PBMC) from patients and controls. The vast majority of PBMC were collected before transplantation. The $(CA)_m(CT)_n$ repeat in the 3' flanking region of the IL-2 gene was determined using polymerase chain reaction (PCR) followed by fragment analysis of the amplified PCR product. For PCR, we used a 5' FAM labeled forward primer (AAA GAG ACC TGC TAA CAC A) and a reverse primer (CCT ATG TTG GAG ATG TTT AT) that covered the dinucleotide repeat. Fragment analysis was performed using an ABI Prism 310 Genetic analyzer with Genescan Analysis software 1.2 (Applied Biosystems). TAMRA 500 (Applied Biosystems) was used as internal size standard.

Statistics

IL-2 allele frequencies were compared between controls and patients, and between patients with and without AR after heart transplantation. Subsequently, we grouped subjects by genotype and distinguished those carrying one or two copies of a particular allele as "carriers" and those not carrying this allele as "non carriers". We compared the genotype frequencies between recipients with and without AR episodes using the (exact) χ^2-test. The strength of association was estimated using logistic regression and expressed as odds ratios (OR) and 95% confidence intervals (95% CI). To test for possible interaction between the IL-2 repeat polymorphism and HLA mismatches, we performed multivariate logistic regression analyses, with both genotype variables in the regression model with an interaction term. Because of the small number of patients in the group with zero HLA-DR mismatches, we combined this group with the one mismatch group in this analysis. P-values ≤ 0.05 were considered significant.

Table 1. Allele distribution of IL-2 dinucleotide repeat polymorphism $(CA)_m(CT)_n$ in the 3'flanking region

alleles	controls (n=101)		patients total (n=290)		patients no AR (n=67)		patients AR (n=223)		p-values no AR vs AR
	alleles	%	alleles	%	alleles	%	alleles	%	
111	**16**	**7.9**	**48**	**8.3**	**13**	**9.7**	**35**	**7.8**	ns
119	0	0	3	0.5	0	0	3	0.7	
121	5	2.5	12	2.1	5	3.7	7	1.6	
123	7	3.4	15	2.6	3	2.2	12	2.7	
125	**22**	**10.9**	**80**	**13.8**	**14**	**10.4**	**66**	**14.8**	ns
127	**18**	**8.9**	**28**	**4.8**	**6**	**4.5**	**22**	**4.9**	ns
129	**29**	**14.5**	**104**	**17.9**	**28**	**20.9**	**76**	**17.0**	ns
131	**11**	**5.4**	**34**	**5.9**	**5**	**3.7**	**30**	**6.7**	ns
133	**32**	**15.8**	**87**	**15.0**	**16**	**11.9**	**70**	**15.6**	ns
135	**20**	**9.9**	**68**	**11.7**	**23**	**17.2**	**45**	**10.1**	0.03
137	**13**	**6.4**	**41**	**7.1**	**8**	**6.0**	**33**	**7.4**	ns
139	**24**	**11.9**	**45**	**7.7**	**10**	**7.5**	**35**	**7.8**	ns
141	4	2.0	14	2.4	3	2.2	11	2.5	
143	1	0.5	0	0	0	0	0	0	
145	0	0	1	0.2	0	0	1	0.2	

no AR: patients without AR and AR: patients with acute rejection. For reasons of statistical power, only alleles with a frequency of more than 5% in our population (bold) were included in the analyses. Overall p-values: p=0.27 (controls vs patients; χ^2-test) and p=0.26 (patients with AR vs patients without AR).

Results

We observed 15 different alleles for the $(CA)_m(CT)_n$ repeat in both the patient and control group. For reasons of statistical power, we only included alleles with a frequency of more than 5% in our population in the analyses.

For the allele distribution, we found no overall significant differences between patients and controls (p=0.27, Table 1) or between patients with and without AR (p=0.26, Table 1) However, the frequency of allele 135 was higher in subjects without AR when compared to those with AR (p=0.03, Table 1).

Also genotype analysis (carrier/non carrier) of allele 135 showed a significant association with freedom from AR after heart transplantation. Recipients without allele 135 had a 14% higher occurrence of AR compared to patients with the 135 allele (p=0.02 figure 1), which corresponded to an Odds Ratio of 2.1 (95% CI: 1.2 to 3.9).

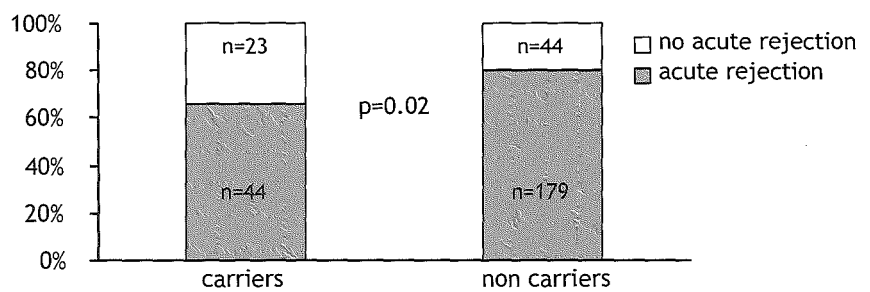

Figure 1. Carriers of the 135 allele of the dinucleotide repeat polymorphism $(CA)_m(CT)_n$ in the 3'flanking region of the IL-2 gene in relation to acute rejection after heart transplantation. The p-value for the difference in distribution was obtained by χ^2-test.

Subsequently, we studied interaction between the 135 allele and HLA-DR mismatching. We observed that recipients without allele 135 rejected their grafts irrespective of the number of HLA-DR mismatches (p=0.86, Table 2). In contrast, in patients with the 135 allele, the frequency of patients with an AR was significantly less when they had 0 or 1 HLA-DR mismatch compared to patients who had 2 mismatches with the donor (p=0.01, table 2). In the regression analysis, we found significant interaction between the presence of allele 135 and the number of HLA-DR mismatches (p=0.02). Our findings were not significantly influenced by the used immunosuppressive regimens, as distribution of allele 135 of the IL-2 repeat was

comparable between patients who did or did not receive anti T-cell induction therapy (135+: n=31 and n=21 vs 135-: n=148 and n=74 respectively; p=0.34, χ^2-test).

Table 2. Interaction of IL-2 dinucleotide repeat polymorphism and HLA-DR matching in relation to AR after heart transplantation

allele	number of HLA-DR mismatches			p-value
	total	0 or 1	2	
135-	179/223 (80%)	85/107 (79%)	94/116 (81%)	0.86
135+	44/67 (66%)	14/29 (48%)	30/38 (79%)	0.01

The numbers are the patients with an AR from the total number of patients in the group. The percentage of rejectors in the groups is given in parentheses. P-values are obtained by χ^2-test.

Discussion

Our findings can be explained by assuming that the studied microsatellite itself is functionally involved in determining genotype-related differences in the development of AR. For example, length polymorphisms in microsatellites have been shown to affect transcription of mRNA and efficiency of translation. Alternatively, we can assume that the 135 allele is genetically linked to a truly functional sequence variation elsewhere in the gene. From the known SNP's in the IL-2 gene, the SNP in the leader sequence (+166) is not of functional significance, since it does not effect the amino acid sequence.[4] The other SNP, in the promoter region of the IL-2 gene at position -330, might be functional. This polymorphism is located within a chromatin regulation site and might influence the chromatin structure, thereby changing the ability of transcription factors to bind and subsequently affecting transcription of IL-2 mRNA.[6] However, conflicting data in regard to an association between the -330 polymorphism and IL-2 production levels have been described. Nieters *et al.* reported no association between this polymorphism and in vitro IL-2 protein production, while Cox *et al.* described higher IL-2 production levels in stimulated peripheral blood lymphocytes from individuals homozygous for the G allele.[7, 8] The complete IL-2 gene is <10 kb, which is within the range of strong linkage disequilibrium in a Caucasian population.[9] We can, therefore, not exclude the possibility that a nearby and functionally related gene that carries a functional polymorphism explains our observation. Thus, in this case, the repeat merely acts as a marker for a functional sequence variation that

remains to be identified.[10] Thus, whether the $(CA)_m(CT)_n$ repeat polymorphism itself is functional or whether it is linked to another functional polymorphism in the IL-2 gene or a nearby gene, needs further investigation.

Previous studies have demonstrated that HLA mismatching is associated with AR after heart transplantation.[11] We also demonstrated in the present study, that the number of HLA-DR mismatches is a risk factor for the development of AR. However, it is clear that HLA mismatches do not completely explain the development of acute rejection, suggesting that additional factors, such as cytokine polymorphisms might play a role. We found an interaction between HLA-DR matching and allele 135 in relation to the development of AR. Apparently, the HLA matching effect on the incidence of AR is only seen in patients who carry the IL-2 $(CA)_m(CT)_n$ repeat allele 135. Our results suggest that HLA matching seems clinically irrelevant in patients without this allele.

We investigated a polymorphism with a large number of alleles and found only allele 135 to be associated to AR after heart transplantation. This association might also be the result of chance, and a Bonferroni correction, in which the critical p-value divided by the number of tests, would be appropriate. However, a disadvantage of such correction is that relevant but minor associations might be overlooked on the basis of the number of alleles and the number of patients studied. In this situation, describing what was done and why and discussing the possible interpretations of each result, should enable the reader to reach a reasonable conclusion without the help of these adjustments.[12] Another statistical approach is to divide the patient group into two subpopulations and then analyze the IL-2 polymorphism in relation to AR in both groups. The association should then be found in both subgroups and in the overall group. Unfortunately, the statistical power of our current study is too low because our patient group is too small to perform such an approach for the 135 allele, which is relatively rare (12%). Therefore, to confirm our preliminary findings, association studies with this polymorphism in other populations are required.

When we consider the IL-2 genotype dependent differences in the development of AR to be true, we can hypothesize a possible mechanism. Individuals without allele 135 of the IL-2 $(CA)_m(CT)_n$ repeat, readily produce IL-2 upon even the slightest donor-specific stimulation. Consequently, they reject their grafts independently of the number of HLA-DR mismatches. In contrast, patients with allele 135 reject their graft only when they are sufficiently stimulated (2 HLA-DR mismatches). In case of less (0 or 1) HLA-DR mismatches, recipients are not sufficiently stimulated to produce IL-2 and, therefore, do not reject their graft.

In conclusion, our findings suggest that HLA-DR matching might only be effective in reducing the AR incidence in the presence of the 135 allele of the dinucleotide repeat polymorphism $(CA)_m(CT)_n$ in the 3' flanking region of the IL-2 gene.

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Chapter 4

Recipient gene polymorphisms in the Th-1 cytokines IL-2 and IFN-γ in relation to acute rejection and graft vascular disease after clinical heart transplantation

Cécile Holweg
Annemiek Peeters
Aggie Balk
André Uitterlinden
Bert Niesters
Lex Maat
Willem Weimar
Carla Baan

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Abstract

IL-2 and IFN-γ are associated with acute rejection (AR) and graft vascular disease (GVD) after clinical heart transplantation. Polymorphisms in the genes of IL-2 (T-330G in the promoter) and IFN-γ (CA repeat in the first intron) influence the production levels of these cytokines. Therefore, these polymorphisms might have an effect on the outcome after transplantation. To investigate possible effects of genetic variations in IL-2 and IFN-γ genes on AR and GVD, we analyzed the IL-2 T-330G and the IFN-γ CA repeat polymorphism in DNA of 301 heart transplant recipients.

No associations were found for allele or genotype distributions between patients with or without AR (IL-2 allele frequency: p=0.44, genotype distribution: p=0.46; IFN-γ allele frequency p=0.10, genotype distribution 12 repeats allele: p=0.21). Also, no associations were found analyzing the number (0 vs 1 vs ≥1) of AR (IL-2 allele frequency: p=0.59; genotype distribution: p=0.37; IFN-γ allele frequency: p=0.27, genotype distribution 12 repeats allele: p=0.41) or analyzing the polymorphisms in patients with AR within the first month or thereafter (IL-2 allele frequency: p=0.45, genotype distribution: p=0.38; IFN-γ allele frequency: p=0.21, genotype distribution 12 repeats allele: p=0.41).

Analyzing both polymorphisms in relation to GVD, resulted in comparable allele and genotype distributions (IL-2 allele frequency: p=0.75; genotype distribution: p=0.77; IFNγ allele frequency: p=0.70, genotype distribution 12 repeats allele: p=0.63).

In conclusion, we did not detect an association between the IL-2 T-330G promoter polymorphism and CA repeat polymorphism in the first intron of the IFN-γ gene and AR or GVD after heart transplantation.

Introduction

Cytokines are proteins or glycoproteins that mediate immune and inflammatory responses and are produced by a variety of cells. They influence the outcome of organ transplantation. Cytokines play a role in acute and chronic allograft rejection and are also thought to be involved in graft acceptance.[1,2] Earlier studies of our group and others have shown that intragraft IL-2 and IFN-γ (Th-1 cytokines) are associated with acute rejection (AR) after heart transplantation. IL-2 mRNA transcripts were more often seen in endomyocardial biopsies (EMB) taken during rejection episodes compared to biopsies taken during a period of quiescence. Also, higher IL-2 and IFN-γ protein levels were found in graft infiltrating lymphocyte cultures derived from EMB with signs of AR than from EMB without rejection.[3-5] In addition, the immunohistochemical study by Ruan *et al.* on EMB showed that IL-2 expression was more prominent in biopsies with severe cellular rejection than in mild rejection and IFN-γ expression increased with the grade of cellular rejection.[3] Furthermore, we showed that IL-2 and IFN-γ are also associated with graft vascular disease (GVD) at one year after heart transplantation. IL-2 mRNA was more often present in the rejection EMB from patients with GVD (77%) than without GVD (33%).[6] Significantly more IL-2 and IFN-γ was produced in graft infiltrating lymphocyte cultures derived from EMB, obtained in the early period after transplantation of patients with GVD compared to patients without GVD diagnosed at 1 year after transplantation.[7] Thus, Th-1 cytokines might be involved in the initiation of GVD after clinical heart transplantation.

The production levels of cytokines differ markedly between individuals, which might be due to polymorphisms in the genes of these immune modulators. Indeed, a number of studies have shown that polymorphisms influence their in vivo and in vitro protein production levels. This also applies to polymorphisms in the genes of IL-2 (T-330G in the promoter) and IFN-γ (CA repeat in the first intron). Hoffmann *et al.* reported that individuals homozygous for the G allele produce more IL-2 after in vitro stimulation with anti-CD3/CD28 than individuals who are heterozygous or homozygous for the T allele.[8] For the IFN-γ CA repeat polymorphism it has been shown that individuals with an allele of 12 repeats produce higher amounts of IFN-γ than individuals with other alleles.[9] A number of association studies of cytokine gene polymorphisms and allogeneic reactivity after organ transplantation have been published. In heart transplantation, associations between polymorphisms in the genes of TNF-α (-308, G→A), IL-10 (-1082, G→A) and IL-2 ($\langle CA\rangle_m\langle CT\rangle_n$ repeat) and AR have been reported.[10-12] However, also studies in which no associations in relation to AR after heart transplantation were found,

are described.[13, 14] Only one study on the CA repeat polymorphism in the IFN-γ gene in relation to AR after heart transplantation has been published. This study by Awad *et al.*, in pediatric heart transplantation did not find an association.[11] In respect to GVD after heart transplantation, a few association studies with polymorphisms in the TGF-ß1 gene (Leu^{10}→Pro and Arg^{25}→Pro) have been reported but these findings are conflicting.[15-17] Until now no studies analyzing the T-330G polymorphism in the IL-2 gene promoter in relation to AR and GVD and for the IFN-γ CA repeat polymorphism in relation to GVD have been reported.

The aim of the present study was to identify patients with a predisposition for AR and GVD after heart transplantation. We investigated whether the high producer alleles of the recipient gene polymorphisms in the Th-1 cytokines IL-2 (T-330G in the promoter; G high producer allele) and IFN-γ (CA repeat in the first intron; 12 repeats high producer allele) are risk factors for AR and GVD after clinical heart transplantation

Materials and methods

Patients and controls

We genotyped DNA of 301 out of 329 consecutive heart transplant recipients, transplanted in Rotterdam between June 1984 and January 1998. From 20 patients, no DNA was available for genotyping and 8 patients died within a few days after transplantation due to non-immunological complications (e.g., surgical complications, infection, cerebral hemorrhage, and multi-organ failure). Ninety-three healthy volunteers served as controls for allele and genotype frequencies. All patients received Cyclosporin A and low-dose of steroids as maintenance immunosuppressive therapy. Cyclosporin A was measured to keep 12 hours trough levels between 250-350 ng/ml in the first 6 months after transplantation, and between 100-200 ng/ml after 6 months.

Both the patient and control population consisted mainly (>95%) of Caucasians. For analyses of the polymorphisms in relation to AR in the first year after heart transplantation, we divided the patient group in patients without (n=63) and with (n=238) rejection episodes. AR was histologically diagnosed in EMB according to the criteria of the International Society for Heart and Lung Transplantation (ISHLT ≥3A).[18]

For analysis of the polymorphisms in relation to GVD, the patient group was divided in patients with (n=85) and without (n=201) GVD. GVD was diagnosed at 1

year after transplantation by visual assessment of the coronary angiogram and defined as all abnormalities including minimal irregularities.[19]

Patient characteristics, i.e., age, gender, primary disease, cold ischemia time, induction therapy, and the number of HLA mismatches, of patients with and without AR and with and without GVD are summarized in Table 1.

Table 1. Characteristics of patients with and without acute rejection episodes in the first post-transplant year and with and without graft vascular disease at one-year after transplantation

	No AR	AR	p-value	No GVD	GVD	p-value
No. of subjects (n)	63	238		201	85	
Age (yr)*	44.9±13.1	47.9±11.0	0.07	46.4±11.7	49.4±10.7	0.04
Gender (m/f)	55/8	198/40	0.79	170/31	71/14	0.87
Primary disease (n)						
Ischemic heart disease	32	129	0.52	103	51	0.18
Dilated cardiomyopathy	30	95		89	28	
Other	2	13		9	6	
Cold ischemic time (min)*	175±41	165±38	0.08	165±38	171±41	0.24
Induction therapy (n)						
No	19	62		56	19	
ATG	17	63	0.54	51	26	0.16
OKT3	21	73		68	22	
BT563	6	40		26	18	
HLA-A+B mismatches (n)*	3.0±0.9	3.0±0.9	0.99	3.0±0.9	2.9±0.9	0.73
HLA-DR mismatches (n)*	1.3±0.7	1.5±0.6	0.03	1.4±0.6	1.5±0.6	0.23

AR: acute rejection, GVD: graft vascular disease. *Mean ± SD. P-values are obtained by (exact) χ^2-test‡ and students t-test‡‡.

DNA isolation

DNA was isolated from 10^6 patient peripheral blood mononuclear cells (PBMC), by the method described by Boom *et al.*[20] The vast majority of PBMC were collected before transplantation. Cells were lysed in a buffer containing triton X-100 and guanidinium-iso-thiocyanate. Thereafter, the DNA was bound to celite, washed twice with a buffer containing guanidinium-iso-thiocyanate, twice with 70% ethanol and once with acetone. The pellet was vacuum dried and dissolved in 100 µl double distillated water. DNA was eluted by incubation at 56°C for 10 min. After centrifugation, the supernatant, containing the genomic DNA, was removed and placed into a clean tube.

Genotyping of the single nucleotide polymorphism T-330G in the promoter of the IL-2 gene

For genotyping of the SNP at position -330 in the promoter region of the IL-2 gene, the TaqMan Allelic Discrimination Assay was used.[21] In short, two probes are used for this polymorphism. Probes are labeled with different 5' reporter dye's (FAM, allele T and VIC, allele G) and a 3'-quencher dye (TAMRA). During amplification, both probes hybridize to a target sequence, whereby the affinity of the probe mismatched for the polymorphism is less than the affinity of the matched probe. DNA polymerase cleaves the bound probes with its 5'-3' nuclease activity and the reporter and quencher dye are separated, resulting in increased fluorescence signal of the reporter. Amplification of DNA was performed using the ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Five µl of DNA was added to 45 µl PCR mixture, containing 2x TaqMan® Universal PCR Master Mix (Applied Biosystems) 0.9 µM of each sequence specific primer (forward: 5'-CTT TTC ATC TGT TTA CTC TTG CTC TTG T-3' and reverse: 5'-TGT ATG AAA CAG TTT TTC CTC CTT TCT-3') and 0.2 µM of each probe, covering the polymorphism (T allele: 5'-FAM-AAA TTT TCT TTG TCA TAA AAC TAC ACT G-TAMRA-3' and G allele: 5'-VIC-AAT TTT CTT TGT CCT AAA ACT ACA CTG-TAMRA-3'). Both probes are turbo probes, i.e., T is replaced by 5-propyne-2' deoxyuridine (Applied Biosystems).

After 2 min at 50°C with uracil *N'*-glycosylase for breaking down carryover PCR products, incubation at 94°C for 10 min was performed to inactivate uracil *N'*-glycosylase and to activate AmpliTaq Gold polymerase. The PCR cycling program consisted of 40 two-step cycles of denaturation at 95°C for 15 s and annealing and extension for 1 min at 58°C. Each PCR run contained 8 negative H_2O controls and 16 positive references (8 for allele T and 8 for allele G). An end point detection is performed to measure the FAM and VIC signal.

Genotyping of the CA repeat polymorphism in the first intron of the IFN-γ gene

Genotyping of the variable length CA repeat in the first intron of the IFN-γ gene was performed by PCR followed by fragment analysis of the amplified PCR product. For PCR, we used a 5' FAM labeled forward primer (5'-FAM-TTA GCT GTT ATA ATT ATA GCT GT-3') and a reverse primer (5'-AGG TTT CTA TTA CAT CTA CTG T-3') covering the CA repeat. The PCR mixture contained 0.2 mM of each dATP, dTTP, dCTP, dGTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.5 µM of each primer and 1 U AmpliTaq GOLD polymerase (Applied Biosystems). Samples were subjected to 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The last cycle was extended with 7 min at

72°C. To confirm amplification, the PCR product was electrophoresed through a 2% agarose gel in 0.5X TBE, containing 0.2 ng/ml ethidium bromide.
Fragment analysis was performed using an ABI Prism™ 310 Genetic analyzer with Genescan™ Analysis software 1.2 (Applied Biosystems). TAMRA 500 (Applied Biosystems) was used as internal size standard.

Statistics
The (exact) χ^2-test was used to compare IL-2 and IFN-γ allele and genotype frequencies, deviation from the Hardy-Weinberg equilibrium, gender distribution, primary disease and treatment with induction therapy. Comparisons of age, cold ischemic time and the mean number of HLA-AB and HLA-DR mismatches were done by Student's *t*-test. $P \leq 0.05$ was considered significant.

Results

Patient characteristics
We studied 301 out of 329 consecutive heart transplant recipients, of who 79 % (238/301) suffered from one or more AR episodes, requiring additional anti-rejection therapy, in the first post transplant year. Six of these patients died due to acute rejection and nine patients died in the first year of other reasons. Of the 289 patients who survived the first year (87%), 85 patients already had signs of GVD at their first year coronary angiogram, while 201 patients remained free from this complication at this time point. Between patients who did (n=238) and did not (n=63) reject their allograft and patients with minor irregularities, by visual analysis (n=85) and without (n=201), we found no differences for recipient gender, underlying disease, cold ischemia time, different induction therapy schemes and HLA A, B mismatches with the donor. The mean number of HLA-DR mismatches was different between patients with and without AR (p=0.03, χ^2-test) and the mean age of patients with GVD at one-year was higher than in patients without GVD (p=0.04, student's *t*-test, Table 1).

Allele and genotype distribution of the IL-2 T-330G gene polymorphism in controls and in cardiac allograft recipients in relation to AR and GVD.
The IL-2 allele and genotype distribution in our control population was similar to reported frequencies by others (allele frequencies: T, 0.71 and G, 0.29; genotype distribution: TT, 51%; TG, 41% and GG, 9%).[22, 23] Also, the allele and genotype distribution of our patient population was similar to the frequencies of our control

Tabel 2. IL-2 (T-330G) single nucleotide polymorphism and IFN-γ CA repeat polymorphism allele frequencies and genotype distribution in heart transplant recipients in relation to acute rejection in the first year after transplantation

	No AR n=63	AR n=238	p-value	No. of AR 0 (n=63)	1 (n=94)	≥2 (n=144)	p-value	Day of 1st AR <31 (n=101)	≥31 (n=87)	p-value
IL-2										
Allele frequencies										
T	97 (0.77)	348 (0.73)	0.44	97 (0.77)	135 (0.72)	213 (0.74)	0.59	144 (0.71)	131 (0.75)	0.45
G	29 (0.23)	128 (0.27)		29 (0.23)	53 (0.28)	75 (0.26)		58 (0.29)	43 (0.25)	
Genotype distribution										
TT	36 (57%)	128 (54%)		36 (57%)	51 (54%)	77 (53%)		53 (52%)	48 (55%)	
TG	25 (40%)	92 (39%)	0.46	25 (40%)	33 (35%)	59 (41%)	0.37	38 (38%)	35 (40%)	0.38
GG	2 (3%)	18 (7%)		2 (3%)	10 (11%)	8 (6%)		10 (10%)	4 (5%)	
IFN-γ										
Allele frequencies										
11 repeats	0 (0)	0 (0)		0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	
12 repeats	66 (0.52)	209 (0.44)		66 (0.52)	79 (0.42)	130 (0.45)		89 (0.49)	67 (0.39)	
13 repeats	50 (0.40)	203 (0.43)	0.10	50 (0.40)	80 (0.42)	123 (0.43)	0.27	75 (0.37)	82 (0.47)	0.43
14 repeats	4 (0.03)	43 (0.09)		4 (0.03)	20 (0.11)	23 (0.08)		18 (0.09)	17 (0.09)	
15 repeats	6 (0.05)	21 (0.04)		6 (0.05)	9 (0.05)	12 (0.04)		10 (0.05)	8 (0.05)	
Genotype distribution										
12 repeats Carriers	50 (79%)	169 (71%)	0.21	50 (79%)	66 (70%)	103 (72%)	0.41	74 (73%)	58 (67%)	0.41
Non carriers	13 (21%)	69 (29%)		13 (21%)	28 (30%)	41 (28%)		27 (27%)	29 (33%)	

P-values are obtained by (exact) χ^2-test.

group (patient allele frequencies: T, 0.74 and G, 0.26; genotype distribution: TT, 54%, TG, 39% and GG, 7%; p-values with controls: p=0.48 and p=0.72, respectively). The found genotype frequencies of both controls and patients were within the Hardy-Weinberg equilibrium.

Next, we analyzed whether the allele frequency and genotype distribution of the IL-2 T-330G polymorphism is associated with AR in the first year after heart transplantation. No differences were found for either the allele frequencies or genotype distribution between patients with and without AR episodes (Table 2). Analyzing this polymorphism in relation to the number of AR episodes resulted also in comparable allele and genotype frequencies (Table 2). Furthermore, no differences were found between the allele or genotype distribution when we studied the polymorphism in patients who rejected their graft within the first month or after one month (Table 2).

When we analyzed the IL-2 T-330G polymorphism in relation to GVD, again similar allele frequencies and genotype distributions were found between patients with and without signs of this complication (Table 3).

Allele and genotype distribution of the IFN-γ CA repeat gene polymorphism in controls and in cardiac allograft recipients in relation to AR and GVD

The IFN-γ allele frequencies in controls were comparable with frequencies reported by Reynard *et al.* (11 to 15 repeats: 0.01, 0.49, 0.39, 0.06, 0.04, respectively).[22] The allele frequencies in our patient population were comparable with the allele frequencies found in our control group (12 to 15 repeats: 0.46, 0.42, 0.08, 0.04; p-value with controls: p=0.75).

The alleles in the patient population were equally distributed between the patients with and without AR episodes in the first year after transplantation. Also no associations were found between the genotype distribution (carriers/non carriers) of the 12 repeats allele (high producer allele) and the occurrence of AR (Table 2). Next, we analyzed the allele and genotype distribution in relation to the number of AR episodes, but again no associations were found for either the allele or genotype distribution (Table 2).

And also analysis in relation to the time of the first acute rejection (i.e., within or after one month after transplantation) resulted in comparable allele and genotype distributions (Table 2).

Finally, we studied the CA repeat polymorphism in the IFN-γ gene in relation to GVD. No differences in allele frequencies or genotype distribution were found between patients with and without GVD at one year (Table 3).

Table 3. Allele frequencies and genotype distribution of the IL-2 (T-330G) and IFN-γ (CA repeat) polymorphisms in relation to graft vascular disease (GVD) at 1 year after transplantation

	No GVD at 1 year n=201	GVD at 1 year n=85	p-value
IL-2			
Allele frequencies			
T	300 (0.75)	124 (0.73)	0.75
G	102 (0.25)	46 (0.27)	
Genotype distribution			
TT	112 (56%)	44 (52%)	0.77
TG	76 (38%)	36 (42%)	
GG	13 (6%)	5 (6%)	
IFN-γ			
Allele frequencies			
11 repeats	0 (0)	0 (0)	0.70
12 repeats	183 (0.45)	78 (0.46)	
13 repeats	175 (0.44)	68 (0.40)	
14 repeats	28 (0.07)	16 (0.09)	
15 repeats	16 (0.04)	8 (0.05)	
Genotype distribution			
12 repeats Carriers	144 (72%)	64 (75%)	0.63
Non carriers	57 (28%)	21 (25%)	

P-values are obtained by (exact) χ^2-test.

Discussion

In the present study, we investigated whether high producer alleles of the recipient IL-2 T-330G promoter polymorphism and the CA repeat polymorphism in the first intron of the IFN-γ gene were associated with the occurrence, incidence or time to the first AR episode and with GVD after clinical heart transplantation. No associations were found for either the IL-2 or the IFN-γ polymorphisms and AR or GVD.

Recently, several other attempts have been made to find associations between cytokine gene polymorphisms and AR or chronic rejection in various transplanted organs.[24] The IL-2 T-330G polymorphism was investigated in relation to AR after renal transplantation, but no association was found.[25] For the IFN-γ CA repeat, Asderakis *et al.* showed that the CA repeat might have an influence on acute rejection in kidney transplant recipients, in particular in patients receiving monotherapy with cyclosporin or those with an HLA-DR mismatched kidney.[26] However, most studies were not able to establish an association between the IFN-γ CA repeat and AR after organ transplantation.[25, 27-29]

In relation to chronic allograft failure, no studies were reported in relation to the IL-2 T-330G polymorphism, while only one association with the CA repeat in the IFN-γ gene has been published. Awad *et al.* reported that patients with allograft fibrosis after lung transplantation carried more often an allele with 12 repeats (the high producer allele) than other alleles.[30]

An explanation for the general lack of an association between the IL-2 T-330G and IFN-γ CA repeat polymorphisms and AR and GVD after organ transplantation can be that the reported correlations between the polymorphisms and in vitro production levels do not apply to the in vivo IL-2 and IFN-γ levels. Or alternatively that possible differences in production levels in vivo are abrogated by the used maintenance immunosuppressive therapy.[8, 9] Furthermore, the effect of the polymorphisms on cytokine production might be overruled by the redundancy of the cytokine network. For example, IL-15 might take over the role of IL-2 in the AR process.[31]

In conclusion, we did not find an association between high producer alleles of the Th-1 cytokine gene polymorphisms IL-2 (T-330G) and IFN-γ (CA repeat) and the occurrence, incidence or time to the first acute rejection episode or graft vascular disease at one year after heart transplantation.

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Chapter 5

TGF-ß1 gene polymorphisms in patients with end-stage heart failure

Cécile Holweg
Carla Baan
Bert Niesters
Pascal Vantrimpont
Paul Mulder
Lex Maat
Willem Weimar
Aggie Balk

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Abstract

The regulatory cytokine transforming growth factor (TGF)-ß1 is thought to play a role in atherosclerotic heart disease as well as in idiopathic cardiomyopathy. The production of TGF-ß1 is genetically controlled as polymorphisms in the signaling sequence of the TGF-ß1 gene Leucine^{10}→Proline and Arginine^{25}→Proline are involved in the regulation of the protein production level. We investigated whether these polymorphisms are associated with end-stage heart failure due to dilated cardiomyopathy (CMP) or ischemic heart disease (IHD).

We determined polymorphisms, using sequence specific oligonucleotide probing (SSOP), in genomic DNA samples from heart transplant recipients (n=253) and controls (n=94). Indications for transplantation were dilated CMP (n=109) and IHD (n=144).

We found a difference in TGF-ß1 codon 10 genotype distribution among patients with IHD, dilated CMP and controls (p=0.034; χ^2-test). Patients with dilated CMP differed from patients with IHD (p=0.044) and healthy controls (0.017). The genotype distribution between patients with IHD and controls was comparable. For codon 25, we found no difference in genotype distribution.

The Leu^{10}→Pro (codon 10) polymorphism in the TGF-ß1 gene is associated with end-stage heart failure caused by dilated CMP and not with IHD. This observation suggests that TGF-ß1 is involved in the pathogenesis of CMP.

Introduction

The involvement of cytokines in the pathogenesis of cardiovascular disease causing heart failure has been extensively demonstrated. Transforming growth factor-ß1 (TGF-ß1), a regulatory cytokine produced by many cell types, has been studied in relation to the pathogenesis of coronary artery disease. Both anti-atherogenic and pro-atherogenic activities of TGF-ß1 have been reported.[1-4] TGF-ß1 inhibits the proliferation of many cells, including smooth muscle cells, endothelial cells and epithelial cells, and could therefore inhibit development of atherosclerosis. Furthermore, it has chemoattractant activities, enhances cell adhesion, and stimulates intra-cellular matrix deposition.[1-4] The contribution of TGF-ß1 in the pathogenesis of cardiomyopathy (CMP) is less extensively studied. Elevated TGF-ß1 gene expression was measured in ventricular biopsies from hypertrophic and dilated CMP hearts, whereas others found decreased TGF-ß1 plasma levels in patients with dilated CMP.[5-7] The contradictory findings in patients with IHD as well as in patients with CMP could result from different biological activities of TGF-ß1 during various stages of both disease processes or to intra-individual variations in TGF-ß1 protein production. The production of TGF-ß1 is genetically controlled.[8-10] In the signaling sequence, two polymorphisms have been identified; 1 at position +869 and 1 at position +915. These polymorphisms result in changes of codon 10 from Leucine to Proline ($Leu^{10}\rightarrow Pro$) and codon 25 from Arginine to Proline ($Arg^{25}\rightarrow Pro$). In particular, the homozygous Arg^{25} genotype has been associated with relatively high in vitro and in vivo TGF-ß1 levels.[8, 10] Analysis of TGF-ß1 polymorphisms showed that the presence of the Arg^{25} allele is associated with increased blood pressure and with the development of graft vascular disease after cardiac transplantation, whereas the Pro^{25} allele was found to be associated with myocardial infarction.[11-13] A recent study, however, could not confirm a relation between these TGF-ß1 polymorphisms and coronary artery disease.[14]
Given the importance of TGF-ß1 in the pathogenesis of various cardiac diseases, we attempted to identify an association between the 2 polymorphisms ($Leu^{10}\rightarrow Pro$ and $Arg^{25}\rightarrow Pro$) in the signaling sequence of the TGF-ß1 gene and end-stage heart failure caused by IHD or dilated CMP.

Materials and methods

Patients

The study population consisted of 267 out of 329 heart transplant recipients from whom peripheral blood mononuclear cells (PBMC) were available. Patients were transplanted in Rotterdam between June 1984 and January 1998. Ninety-four healthy volunteers served as controls. Patients and controls (n=94) consisted mainly (>95%) of Caucasians. Indications for heart transplantation were dilated CMP (n=109), IHD (n=144) and primary valvular disease (n=14). Because of the small number, patients with primary valvular disease were not analyzed. All patients were in New York Heart Association class III or IV at time of transplantation and without contraindications for heart transplantation, i.e., infection, irreversible pulmonary hypertension, severe kidney or liver dysfunction, diabetes mellitus with secondary organ damage, other systemic disease or severe peripheral vascular disease.

DNA isolation

We isolated DNA from 10^6 PBMC, by the method described by Boom *et al.*[15] We collected 91 percent (243/267) of the PBMC were collected before transplantation. To exclude the influence of donor cells on the outcome of the results in samples taken after transplantation, we tested samples from 40 individuals taken both before and after transplantation. Genotypes before and after transplantation were exactly the same. We lysed PBMC in a buffer containing triton X-100 and guanidinium-iso-thiocyanate and bound the DNA to celite. We washed it twice with a buffer containing guanidinium-iso-thiocyanate, twice with 70% ethanol and once with acetone. The pellet was vacuum dried and dissolved in 100 µl bidest. We eluted the DNA by incubation at 56°C for 10 minutes. After centrifugation, the supernatant, containing the genomic DNA, was removed and placed into a clean tube.

DNA amplification

DNA was amplified using polymerase chain reaction (PCR) in a GeneAmp PCR system 9600 (PE Biosystems, Norwalk, CT, USA). We performed PCR with sequence specific primers covering the first exon of the TGF-ß1 gene, located on chromosome 19q13.1 (Genome Database, accession no GDB 624780).[8] Two µl DNA was added to 28 µl PCR mixture containing 0.2 mM of each dATP, dTTP, dCTP, dGTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 M betaine monohydrate, 0.5 µM of each primer and 1 U AmpliTaq GOLD polymerase (PE

Biosystems). After a 10 min 94°C enzyme activation, samples were subjected to 40 cycles of respectively denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 minute. The last cycle was extended with 7 min at 72°C. To confirm amplification, the PCR product was electrophoresed through a 2% agarose gel in 0.5X TBE, containing 0.2 ng/ml ethidium bromide.

Detection of TGF-ß1 polymorphisms by Sequence Specific Oligonucleotide Probing (SSOP)

The studied TGF-ß1 polymorphisms (+869, $Leu^{10}\rightarrow Pro$ and +915, $Arg^{25}\rightarrow Pro$) were determined by dot blot hybridization. Two biotinilated oligonucleotide probes were used to determine each polymorphism.[8] Two µl PCR product was spotted onto Hybond™-N+ membrane (Amersham Pharmacia, Buckinghamshire, UK) and treated with 0.5 M NaOH and 1.5 M NaCl for 5 min to separate double stranded amplified DNA, followed by a neutralization step with 1.5 M NaCl and 0.5 M Tris, pH 7.5 for 1 minute. The membranes were baked in a microwave for 5 min and DNA was immobilized onto the membranes by crosslinking with UV for 1 minute. Thereafter blots were incubated in 10 ml of hybridization buffer containing 5X SSC, 0.5X Denharts solution, 0.2 M EDTA, 0.5% SDS and 0.1 ml sonicated herring sperm (Promega, Madison, Wisconsin, USA) at 42.5°C for 30 minutes. We added 400 ng of specific biotinilated oligonucleotide probe and allowed to hybridize for 90 min at 42.5°C. The membranes were washed twice with 5X SSC and 0.1% SDS at room temperature for 5 min, followed by stringency washing with 1X SSC and 0.1% SDS at 58°C (+869, $Leu^{10}\rightarrow Pro$) and 61°C (+915, $Arg^{25}\rightarrow Pro$) for 30 minutes. Before visualizing the hybridized probes, the membranes were washed in 0.15 M NaCl and 0.1 M Tris buffer pH 7.5 for 1 minute and treated with 0.5% blocking agent (Roche Diagnostics, Almere, The Netherlands) for 30 minutes. Subsequently, the membranes were incubated with a streptavidine horseradish labeled peroxidase conjugate (Amersham Pharmacia), for 30 min at room temperature before detection by chemoluminiscence using the ECL™ system (Amersham Pharmacia). We determined TGF-ß1 genotypes of patients and controls in two replicate experiments. Figure 1 shows an autoradiogram of a typical example for the TGF-ß1 codon 10 and 25 genotypes.

Statistics

We used the (exact) χ^2-test to compare genotype frequencies and gender distribution between the groups. If the exact χ^2-test was used, then the exact mid p-value was calculated, using StatXact software (CYTEL Software corporation, Cambridge, MA, USA). For testing the genotype frequencies, three pairwise

comparisons were done, if overall p <0.05. For each of the three pairwise comparisons, significance is supposed to be reached if the p <0.02. We compared among the different groups using one way analysis of variance (ANOVA). P-values ≤0.05 were considered significant.

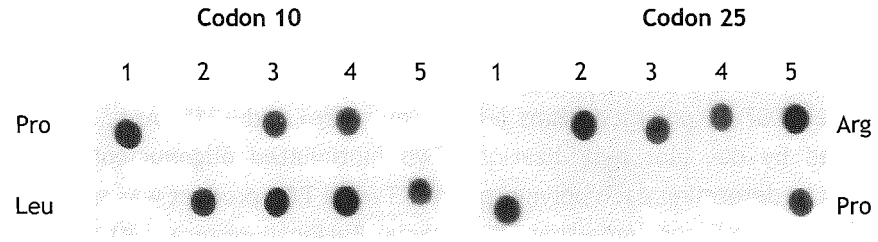

Figure 1. Autoradiogram of an example for TGF-ß1 codon 10 and codon 25 genotypes. For codon 10, individual 1 is homozygous Proline; 2 and 5 are homozygous Leucine and 3 and 4 are heterozygous. For codon 25, individual 1 is homozygous Proline; 2, 3 and 4 are homozygous Arginine and 5 is heterozygous.

Results

Table 1 summarizes patient and control demographics. The mean age differed significantly among the 3 groups (patients with IHD were older than patients with CMP, while these patients were older than controls, p<0.01, ANOVA). We also found a significant difference in gender distribution among these groups (p<0.01, chi-square-test). We found no relation between TGF-ß1 genotype distribution and age or gender in either the patient or control groups (p>0.05).

Table 1. Patient and control demographics

Disease	CMP	IHD	Controls	p-value
No. of subjects	109	144	94	
Age*	43 ± 13.7	50.8 ± 7.7	36.7 ± 10.3	<0.01†
Male/female	82/27	135/9	49/45	<0.01‡

CMP: cardiomyopathy; IHD ischemic heart disease. *Years are expressed as mean ± SD. P-values were obtained by †one-way ANOVA and ‡χ^2-test. P-values apply for differences between all 3 groups.

When we analyzed the TGF-ß1 polymorphisms in relation to the original disease of the cardiac allograft recipients, we found a different genotype distribution for codon 10 (Leu^{10}→Pro) among patients who were transplanted for end-stage dilated CMP, patients in whom IHD was the cause of heart failure and controls (p=0.034, exact χ^2-test). The dilated CMP patient group, had a different TGF-ß1 genotype frequency compared with the patient group with IHD (p=0.044, Table 2) and compared with healthy controls (p=0.017, Table 2). We found no difference between the patients with IHD and controls. This finding suggests that end-stage heart failure caused by dilated CMP is associated with a particular TGF-ß1 genotype. For codon 25 (Arg^{25}→Pro), the genotype frequency was not associated with one of the cardiac diseases, IHD or dilated CMP.

Table 2. Distribution of TGF-ß1 genotypes for codon 10 and 25 in patients and controls

TGF-ß1 genotype	CMP (n=109) n (%)	IHD (n=144) n (%)	Controls (n=94) n (%)	p-value
Codon 10				
Leu/Leu	32 (29.4)	60 (41.7)	37 (39.4)	
Leu/Pro	70 (64.2)	70 (48.6)	43 (45.7)	0.034*
Pro/Pro	7 (6.4)	14 (9.7)	14 (14.9)	
Codon 25				
Arg/Arg	93 (85.3)	123 (85.4)	79 (84.0)	
Arg/Pro	15 (13.8)	18 (12.5)	15 (16.0)	0.631*
Pro/Pro	1 (0.9)	3 (2.8)	0 (0)	

CMP: cardiomyopathy; IHD: ischemic heart disease. P-values were obtained by exact χ^2-test. *: Overall exact mid p-values for TGF-ß1 genotype distribution for codon 10 and 25. If overall p-value <0.05, pairwise comparisons were done. Codon 10: IHD vs controls, p=0.512; IHD vs CMP, p=0.044; CMP vs controls, p=0.017.

Discussion

We studied 2 polymorphisms in the signal sequence of the TGF-ß1 gene in PBMC derived from patients who were transplanted because of end-stage heart disease caused by dilated CMP or IHD, and controls. Our results show that patients who were transplanted because of heart failure due to dilated CMP had a different TGF-ß1 genotype distribution for Leu^{10}→Pro, and not for Arg^{25}→Pro, compared with patients with IHD and controls. The TGF-ß1 genotype distributions were in the Hardy-Weinberg equilibrium, except for codon 10 of the CMP patients, in which

more heterozygotes were observed. An excess of heterozygotes may indicate the presence of overdominant selection.

Protein synthesis is post-translationally regulated by signal sequences. Therefore, polymorphisms in these signal sequences may affect the protein production. The change from a hydrophobic Leucine to a less hydrophobic Proline in codon 10 of the TGF-ß1 gene alters the protein production by impairing the ability to cross the endoplasmic reticulum. Indeed, Awad *et al.* reported an association between Leucine at codon 10 and elevated circulating TGF-ß1 levels in patients with cystic fibrosis.[8] But, a recent report by Yamada *et al.*, showed that TGF-ß1 serum levels were the highest in individuals with Proline at codon 10.[16] The mutation in codon 25 from Arginine to Proline, has an effect on the nearby cleavage side and complicates the release of the TGF-ß1 protein from the cell. A clear-cut link between homozygous Arg^{25} and high TGF-ß1 protein production in vitro was found.[8, 10] Evidence suggests that higher TGF-ß1 protein levels protect against the development of atherosclerosis.[3, 4, 17] Patients with advanced atherosclerosis had depressed circulating levels of TGF-ß1 compared to controls with normal coronary arteries.[3, 4] Atherosclerosis is an inflammatory disease, in which macrophages, monocytes, endothelial cells and smooth muscle cells are involved. In vitro and in vivo studies have shown that TGF-ß1, an anti-inflammatory cytokine, can inhibit the proliferation and migration of vascular smooth muscle cells, thereby preventing the formation of fatty streaks, the first lesions in the development of atherosclerotic plaques.[1, 4, 14, 18] Every individual develops atherosclerotic lesions to some extent, and this may explain our findings that IHD patients and controls have a comparable TGF-ß1 genotype distribution. In contrast, coronary arteries of patients with dilated CMP are relatively free from atherosclerosis. The codon 10 TGF-ß1 genotype distribution, and consequently the TGF-ß1 production capacity, of these patients may differ from that in patients with end-stage IHD and healthy controls. Higher local TGF-ß1 mRNA levels have previously been found in patients with dilated CMP compared to patients with mild global ventricular dysfunction.[6] These elevated levels of TGF-ß1 may lead to remodeling of myocardial matrix seen in dilated CMP.[6] On the other hand, increased TGF-ß1 protein levels in patients with dilated CMP are not uniformly reported.[7] There are several explanations for these contrasting findings. First, TGF-ß1 levels in the peripheral blood may not always reflect the true cytokine burden in the failing heart. Second, these TGF-ß1 levels represent the net result of the balance between a number of pro-inflammatory and anti-inflammatory cytokines. This balance is disturbed in patients with heart failure.[7, 19, 20] Also differences in medication and in TGF-ß1 production by non-myocardial tissues may contribute to the outcome of circulating

protein measurements. Furthermore, careful plasma collection is of great importance, as degranulation of platelets falsely raises the TGF-ß1 levels.[21, 22] TGF-ß1 gene polymorphisms reflect the intrinsic production capacity and measurements are therefore not biased by the above mentioned factors.

In conclusion, the TGF-ß1 (Leu^{10}→Pro) genotype is associated with patients with end-stage heart failure caused by dilated CMP. Although other cytokines are involved, our findings suggest that TGF-ß1 is also a factor in the pathophysiology of dilated CMP.[23] The difference in TGF-ß1 gene polymorphism distribution in the patient group with dilated CMP needs further investigation. This group is of interest because a difference may exist between patients with hereditary CMP and patients with CMP caused by toxic agents or viral infection. It might also be interesting to investigate whether an association exists between TGF-ß1 gene polymorphisms and the progression or severity of cardiac dysfunction.

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Chapter 6

The transforming growth factor-ß1 codon 10 gene polymorphism and accelerated graft vascular disease after clinical heart transplantation

Cécile Holweg
Carla Baan
Aggie Balk
Bert Niesters
Lex Maat
Paul Mulder
Willem Weimar

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Abstract

The multifunctional cytokine Transforming Growth Factor (TGF)-ß1 is thought to play a role in the pathogenesis of graft vascular disease (GVD). Polymorphisms at codon 10, (Leu^{10}→Pro) and codon 25 (Arg^{25}→Pro) in the signal sequence of the TGF-ß1 gene regulate the production and secretion of the protein. We investigated whether these polymorphisms are risk factors for the development of GVD after clinical heart transplantation.

TGF-ß1 polymorphisms, Leu^{10}→Pro and Arg^{25}→Pro, were determined in DNA from heart transplant recipients (n=252) and their donors (n=213), using sequence-specific oligonucleotide probing. GVD was angiographically diagnosed 1-year after transplantation. In addition other potential risk factors including underlying disease, recipient and donor age, recipient and donor gender, number of acute rejections in the first year, cold ischemia time and HLA mismatches were analyzed by univariate and multivariate logistic regression analysis.

Univariate analysis showed that the recipient TGF-ß1 polymorphism Leu^{10}→Pro, ($p=0.056$, χ^2-test), underlying disease ($p=0.01$, χ^2-test), number of acute rejections in the first-year ($p=0.03$, analysis of variance), and donor age ($p<0.001$, analysis of variance) were risk factors for the development of GVD. The TGF-ß1 Arg^{25}→Pro polymorphism was not a risk factor. Also in the multivariate analysis, the recipient TGF-ß1 codon 10 polymorphism was associated with GVD, with patients homozygous for Pro at greatest risk (odds ratio 7.7, $p=0.03$). Apart for the recipient TGF-ß1 Leu^{10}→Pro polymorphism, donor age appeared to be an independent risk factor for the development of GVD at 1-year. Patients with older donor hearts were at greater risk than patients receiving grafts from younger donors (odds ratio 1.1 per year, $p<0.001$).

Recipient TGF-ß1 Leu^{10}→Pro polymorphism and higher donor age are independent risk factors for the development of GVD after clinical heart transplantation.

Introduction

Long-term survival after heart transplantation is influenced by graft failure due to graft vascular disease (GVD). In human cardiac allograft recipients, GVD is a process of accelerated coronary artery disease. This type of chronic rejection is characterized by progressive and diffuse concentric fibrosis and smooth muscle cell proliferation with collagen accumulation leading to intimal thickening of the entire length of donor coronary vessels.[1] Various allogen-dependent factors, like HLA matching, cytomegalovirus (CMV) infection, severity, number and nature of acute rejection episodes, and allogen-independent factors, like donor and recipient age, cold ischemia time, reperfusion, brain death, hyperlypidemia, hypertension and underlying heart disease of the recipient are thought to contribute to the development of GVD.[2-8] These factors can affect endothelial cells resulting in their activation and subsequently to the production of cytokines including platelet derived growth factor (PDGF)-α, basic fibroblast growth factor (bFGF) and transforming growth factor (TGF)-ß1, which are involved in the intimal thickening.[1] TGF-ß1 regulates the proliferation and differentiation of cells, embryonic development, wound healing and angiogenesis.[9, 10] Clearly TGF-ß1 plays a central role in processes in which cells and tissues respond to injury and initiate repair. It is a chemoattractant for lymphocytes, granulocytes and fibroblasts to the place of injury and enhances extracellular matrix production by fibroblasts and other cells.[11, 12] Therefore, a role for TGF-ß1 in the development of GVD after heart transplantation can be envisaged. However, TGF-ß1 is a potent inhibitor of cell proliferation, including endothelial cells, smooth muscle cells and lymphocytes.[12-14] Thus, TGF-ß1 may function as an inhibitor of atherosclerosis and it also has immunosuppressive properties.[9, 14] These are arguments against an initiating role for TGF-ß1 in the development of GVD after heart transplantation. However, these may not be valid under circumstances of immunosuppressive therapy with calcineurine phosphatase blockers, antimetabolites and steroids. To make it more complex, the activities of TGF-ß1 are dependent on the responding cell type, the presence of other growth factors and the local TGF-ß1 level.[14] This level is the result of the individual production and secretion capacity for TGF-ß1, which is genetically controlled.[15-17] In the signaling sequence of the TGF-ß1 gene, two polymorphisms in the first exon have been described, at position +869 and at position +915 relative to the first transcription startpoint. These polymorphisms result in changes of codon 10 from Leucine to Proline (Leu^{10}→Pro) and of codon 25 from Arginine to Proline (Arg^{25}→Pro). Associations of the TGF-ß1 codon 10 and codon 25 genotypes in relation to dilated cardiomyopathy, transplant related

coronary artery disease, blood pressure and myocardial infarction have been described.[18-20]

To investigate whether the gene polymorphisms (Leu^{10}→Pro and Arg ^{25}→Pro) in the signal sequence of the TGF-ß1 gene are risk factors for the development of GVD, we determined both polymorphisms in DNA samples from heart transplant recipients and their accompanying donors. GVD was angioghraphically diagnosed 1 year after transplantation. We analyzed the TGF-ß1 gene polymorphisms in a multivariate analysis, together with other potential risk factors for GVD.

Materials and methods

Patients

From 252 out of 329 consecutive heart transplant recipients, DNA samples were available for TGF-ß1 genotyping. These patients were transplanted between 1984 and 1998. In addition, we studied 213 accompanying heart donors. In patients surviving the first year (236 out of 252, and 192 out of 213 donor hearts), GVD was diagnosed by visual assessment of the coronary angiogram. GVD was defined as all abnormalities, including minimal irregularities.[21] At the 1-year time point, 72 patients were positive and 164 patients were negative for GVD. Of the donor hearts, 62 had signs of GVD and 135 were free of this disease. Characteristics of the two patient groups are summarized in table 1. The vast majority of the individuals studied were Caucasians: patients 98% and donors 100%.

Table 1. Characteristics of patients with and without accelerated graft vascular disease (GVD) at 1 year

	GVD - (n=164)	GVD + (n=72)	p-value
Recipient age (years)	46.4±11.6	49.3±11.1	0.072*
Donor age (years)	24.7±8.5	31.1±9.4	<0.001*
AR 1st year (n)	1.5±1.4	2.0±1.8	0.03*
Primary diagnosis (n)			
IHD	85	50	0.01‡
CMP	79	22	
Cold ischemia time (min)	149±58	159±60	0.23*
Recipient gender (m/f)	142/22	61/11	0.70‡
Donor gender (m/f)	104/60	45/27	0.89‡
HLA mm AB (n)	3.0±0.9	2.9±0.9	0.32*
HLA mm DR (n)	1.5±0.6	1.6±0.6	0.22*

Mean ± SD. P-values are obtained by * one-way ANOVA and ‡ χ^2-test.

We analyzed the following risk factors for the early development of GVD in a multivariate analysis, underlying disease (12 patients with primary valvular disease were excluded due to small numbers), recipient and donor age, recipient and donor gender, cold ischemia time, number of HLA mismatches, the number of acute rejection episodes in the first year, and recipient and donor TGF-ß1 genotypes for codon 10 and codon 25. In the multivariate analysis, the number of patients was only slightly limited by incompleteness of the dataset (n=231).

DNA isolation

DNA was isolated from 10^6 patient PBMC and donor PBMC or spleen cells, by the method described by Boom *et al.*[22] The vast majority of peripheral blood mononuclear cells (PBMC) were collected before transplantation. Cells were lysed in a buffer containing triton X-100 and guanidinium-iso-thiocyanate and the DNA was bound to celite, washed twice with a buffer containing guanidinium-iso-thiocyanate, twice with 70% ethanol and once with acetone. The pellet was vacuum dried and dissolved in 100 µl double distillated water. DNA was eluted by incubation at 56°C for 10 min. After centrifugation, the supernatant, containing the genomic DNA, was removed and placed into a clean tube.

DNA amplification

DNA was amplified using polymerase chain reaction (PCR) in a GeneAmp PCR system 9600 (PE Biosystems, Norwalk, CT, USA). PCR was performed with sequence specific primers for TGF-ß1 covering the first exon.[15] Two µl DNA were added to 28 µl PCR mixture containing 0.2 mM of each dATP, dTTP, dCTP, dGTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 M betaine monohydrate, 0.5 µM of each primer and 1 U AmpliTaq GOLD polymerase (PE Biosystems). After a 10 min 94°C enzyme activation, samples were subjected to 40 cycles of respectively denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. The last cycle was extended with 7 min at 72°C. To confirm amplification, the PCR product was electrophoresed through a 2% agarose gel in 0.5X tris borate EDTA, containing 0.2 ng/ml ethidium bromide.

Detection of TGF-ß1 polymorphisms by sequence specific oligonucleotide probing (SSOP)

The studied TGF-ß1 polymorphisms (+869, Leu^{10}→Pro and +915, Arg^{25}→Pro) were determined by dot blot hybridization. Two biotinilated oligonucleotide probes were used to determine each polymorphism.[15] Two µl PCR product were spotted onto Hybond™-N+ membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK)

and was treated with 0.5M NaOH and 1.5 M NaCl for 5 min to separate double stranded amplified DNA, followed by a neutralization step with 1.5 M NaCl and 0.5 M Tris, pH 7.5 for 1 min. The membranes were baked in a microwave for 5 min and DNA was immobilized onto the membranes by crosslinking with UV for 1 min. Blots were incubated in 10 ml of hybridization buffer containing 5X SSC, 0.5X Denharts solution, 0.2 M EDTA, 0.5% SDS and 0.1 ml sonicated herring sperm (Promega, Madison, Wisconsin, USA) at 42.5°C for 30 min. Four hundred ng of specific biotinilated oligonucleotide probe was added and allowed to hybridize for 90 min at 42.5°C. The membranes were washed twice with 5X SSC and 0.1% SDS at room temperature for 5 min, followed by stringency washing with 1X SSC and 0.1% SDS at 58°C (+869, Leu^{10}→Pro) and 61°C (+915, Arg^{25}→Pro) for 30 min. Before visualizing the hybridized probes, the membranes were washed in 0.15 M NaCl and 0.1 M Tris buffer pH 7.5 for 1 min and treated with 0.5% blocking agent (Roche Diagnostics, Almere, The Netherlands) for 30 min. Subsequently, the membranes were incubated with a streptavidine horse radish labeled peroxidase conjugate, (Amersham Pharmacia Biotech) for 30 min at room temperature before detection by chemoluminiscence using the ECL system (Amersham Pharmacia Biotech).

Statistics

The (exact) χ^2-test was used to compare TGF-ß1 genotype frequencies, deviation from the Hardy-Weinberg equilibrium, gender distribution and distribution of indications for transplantation. If the exact χ^2-test was used, then the exact mid p-value was calculated, using StatXact software (CYTEL Software corporation, Cambridge, MA, USA). Comparison of age, number of acute rejections in the first year and number of HLA mismatches were done by one-way analysis of variance (ANOVA). To identify risk factors for the early development of GVD, multivariate logistic regression analysis was performed. P-values ≤ 0.05 were considered significant.

Results

In the studied patient group, 72 out of 236 (31%) patients had developed early signs (at 1 year) of GVD after transplantation. We found the survival of cardiac allograft recipients to be related with this early diagnosis of GVD. The median survival of patients with GVD after 1 year was significantly worse compared to those patients without angiographic signs of GVD (9.9 vs. 11.7 years; $p<0.0001$; Logrank test, Fig. 1).

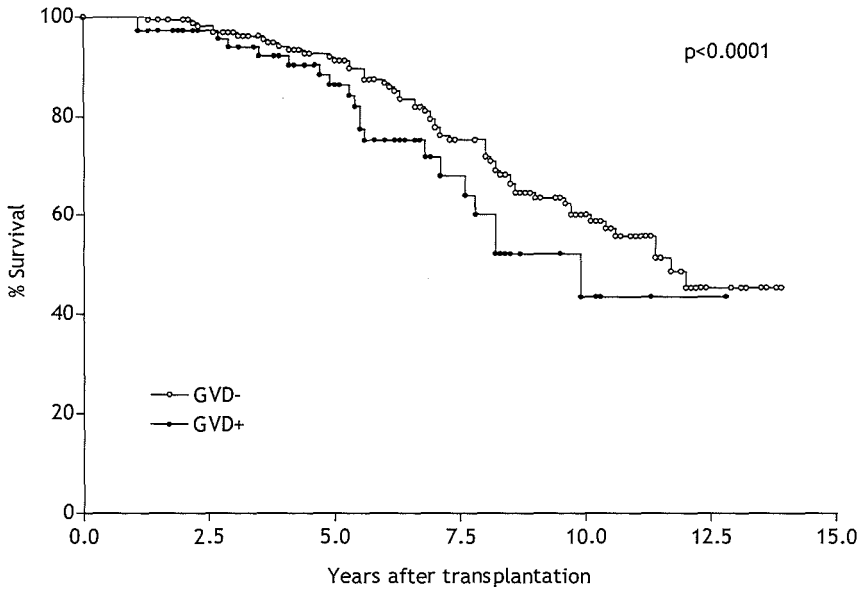

Figure 1. Kaplan Meier survival curve of heart transplant recipients with (n=72) and without (n=164) graft vascular disease at one year after transplanatation. The median survival of recipients with GVD is 9.9 years vs 11.7 years without GVD.

Univariate analysis of the TGF-ß1 codon 10 and 25 genotype distribution in relation to the development of graft vascular disease for both the recipient and the donor are shown in Table 2. A difference was found in the distribution for the TGF-ß1 codon 10 genotype of the recipient (p=0.056, χ^2-test), but not for codon 25 (p=0.254) between patients with and without GVD. In contrast, donor genotypes, both for codon 10 and codon 25, were not associated with early development of GVD (p=0.886 and p=1.0, respectively). Furthermore, the underlying disease, number of acute rejections in the first post operative year and donor age were associated with GVD (p=0.01, χ^2-test; p=0.03 and p<0.001, ANOVA, respectively)
To determine whether TGF-ß1 gene polymorphisms were indeed a risk factor for the development of the accelerated form of GVD, a multivariate logistic regression analysis was performed. Factors included were recipient and donor age, gender of the recipient and donor, underlying disease, duration of cold ischemia time, HLA mismatches, AR episodes during the first year (Table 3). This multivariate analysis confirmed that the TGF-ß1 codon 10 gene polymorphism was an independent significant risk factor for the development of GVD (p=0.03; Table 3). In particular patients with the homozygous Pro genotype were at risk (odds ratio: Pro/Pro, 7.7; Leu/Pro, 1.6 and Leu/Leu, 1.0, Table 3).

Table 2. Univariate analysis of TGF-ß1 codon 10 and codon 25 genotypes in relation to graft vascular disease at 1 year

	GVD - n (%)	GVD + n (%)	p-value
Recipient TGF-ß1	(n=164)	(n=72)	
Codon 10			0.056
Leu/Leu	63 (38%)	25 (35%)	
Leu/Pro	93 (57%)	37 (51%)	
Pro/Pro	8 (5%)	10 (14%)	
Codon 25			0.254
Arg/Arg	136 (83%)	65 (90%)	
Arg/Pro	25 (15%)	7 (10%)	
Pro/Pro	3 (2%)	0 (0%)	
Donor TGF-ß1	(n=135)	(n=62)	
Codon 10			0.886
Leu/Leu	50 (37%)	24 (39%)	
Leu/Pro	64 (47%)	30 (48%)	
Pro/Pro	21 (16%)	8 (13%)	
Codon 25			1.0
Arg/Arg	118 (87%)	54 (87%)	
Arg/Pro	16 (12%)	8 (13%)	
Pro/Pro	1 (1%)	0 (0%)	

P-values are obtained by the (exact) χ^2-test.

Also donor age but none of the other analyzed risk factors of non-immunological (recipient and donor age, gender of the recipient and donor, underlying disease, duration of cold ischemia time) and of immunological origin (HLA mismatches, number of AR episodes during the first year) proved to be associated with early development of GVD.

Table 3. Multivariate logistic regression analysis of risk factors for the early development of graft vascular disease (GVD) at 1 year

	Odds ratio	p-value
Recipient TGF-ß genoype		
Codon 10		0.03
Leu/Leu	1.0	
Leu/Pro	1.6	
Pro/Pro	7.7	
Codon 25		0.09
Underlying disease		0.09
Recipient age		0.84
Donor age	1.1*	<0.001
Recipient gender		0.50
Donor gender		0.65
Number of AR 1st year		0.10
Cold ischemia time		0.19
HLA AB mismatches		0.25
HLA DR mismatches		0.19

* Odds ratio is 1.1 per year.

Discussion

We studied whether TGF-ß1 gene polymorphisms in codon 10 (Leu^{10}→Pro) and codon 25 (Arg^{25}→Pro) are a risk factor for accelerated development of GVD in both an univariate and a multivariate analysis. Apart from TGF-ß1 gene polymorphisms, the multivariate analysis included HLA mismatches and number of acute rejection episodes, underlying disease, recipient and donor age, recipient and donor gender and cold ischemia time. CMV disease and triglyceride levels were not included in the analysis. As we previously showed that these two factors did not significantly contribute to the development of accelerated GVD in our patient population.[21]

The univariate analysis showed a difference in distribution of the recipient TGF-ß1 codon 10 (Leu^{10}→Pro) genotype between patients with and without early signs of GVD at 1 year. The genotype frequencies matched the Hardy-Weinberg equilibrium, except for the patient codon 10 genotype of the GVD negative group. We observed an excess of heterozygotes, which may indicate the presence of

overdominant selection. Indeed, we have described previously an association between TGF-ß1 codon 10 polymorphism and cardiomyopathy as indication for transplantation.[18]

The multivariate analysis showed that in our patient population, both higher donor age and TGF-ß1 codon 10 genotype are independent risk factors for the early development of GVD. The relative risk of the donor age to the development of accelerated GVD increases with 1.1 per year and individuals with the homozygous Proline genotype have a 7.7 times greater risk compared to individuals who are heterozygous or homozygous for Leucine.

The higher donor age in patients with accelerated GVD confirms our data of two univariate studies and results published by others.[8, 21, 23, 24] An explanation for these findings can be that older donor hearts have relatively more pre-existing atherosclerosis than hearts from younger donors. Abnormalities seen at the first year coronary angiogram may have developed in the donor, before transplantation. Thus, our analysis revealed just one other risk factor for the development of GVD apart for donor age: the TGF-ß1 codon 10 gene polymorphism. In cardiac allograft recipients TGF-ß1 (codon 10) proline might be a risk factor for the development of accelerated GVD. Recently Aziz described an association between cardiac vasculopathy, high intragraft TGF-ß1 protein expression and TGF-ß1 codon 25 genotypes.[25] However, others and we were not able to confirm these data. Difference in genetic background, immunosuppressive protocols, timing, and definition of graft vascular disease might, in part, explain the discrepancy between the various studies.[25, 26]

Polymorphisms in the TGF-ß1 gene regulate the level of production and secretion. There are conflicting data whether individuals with the Proline allele produce higher or lower amounts of the TGF-ß1 protein. Yamada et. al. showed that individuals with the proline allele are the high producers.[27] This observation is supported by the data reported by Grainger who showed that TGF-ß1 Pro (10) is in linkage disequilibrium with the -509 T promoter polymorphism that has been associated with high TGF-ß plasma concentrations.[16] However, studies in transplant settings did not confirm these findings. Lung allograft recipients with the Leucine allele at codon 10 produced the highest amounts of TGF-ß1.[15] Therefore it is not clear whether the association between Proline allele in codon 10 and GVD is the result of a high or low production capacity for TGF-ß1. However, it has been shown that TGF-ß1 levels are low in patients with advanced coronary artery disease. This suggests that TGF-ß1 plays a beneficial role and that high levels might protect against atherosclerosis.[13] Low TGF-ß1 production could be associated with chronic rejection associated coronary artery disease after clinical

heart transplantation as well. This postulation seems to be in contrast with the development of chronic rejection after lung transplantation, where high levels of TGF-ß1 are a significant risk factor.[17] However, the processes of chronic rejection after lung transplantation (i.e., obliterative bronchiolitis) might be mediated by one of the other properties of TGF-ß1. TGF-ß1 enhances the production of the extracellular matrix, which is one of the characteristics of chronic rejection after lung transplantation.[12] However, chronic rejection after heart transplantation is dominated by proliferation of smooth muscle cells, which is inhibited by the multifactorial cytokine TGF-ß1.[13] Thus, high TGF-ß1 production after heart transplantation slows down the process of chronic rejection while high TGF-ß1 production after lung transplantation accelerates obliterative bronchiolitis.
Our data support the hypothesis that TGF-ß1 is involved in the development of GVD. We speculate that individuals with the Pro (10) allele are the low producers for TGF-ß1 and are therefore at higher risk for the accelerated development of coronary artery disease after clinical heart transplantation. Prevention of endothelial damage therefore, should be special targeted in these patients and they should be monitored cautiously for early signs of GVD.

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Chapter 7

Renal failure after clinical heart transplantation is associated with the TGF-ß1 codon 10 gene polymorphism

Carla Baan
Aggie Balk
Cécile Holweg
Iza van Riemsdijk
Lex Maat
Pascal Vantrimpont
Bert Niesters
Willem Weimar

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Abstract

To determine whether genetic factors are involved in the development of renal dysfunction due to cyclosporin nephrotoxicity, we analyzed 2 polymorphisms in the signal sequence of the transforming growth factor (TGF)-ß1 gene; codon 10 ($Leu^{10}\rightarrow Pro$) and codon 25 ($Arg^{25}\rightarrow Pro$).

Using sequence specific oligonucleotide probing, we analyzed both TGF-ß1 gene polymorphisms in cardiac allograft recipients (n=168) who survived at least 1 year with a minimal follow-up of 7 years. Patients received cyclosporine and steroids as maintenance immunosuppressive therapy. Renal dysfunction was defined as a serum creatinine ≥250 µmol/l.

Renal dysfunction was observed in 2% (3/168) of the patients at 1-year, in 7% (11/160) at 3-year, in 12% (18/152) at 5-year, and in 20% (26/131) at 7-year post-transplantation. The genotypic distributions for TGF-ß1 codon 10 were: 7% Pro/Pro, 61% Pro/Leu, and 32% Leu/Leu, and for codon 25 these percentages were 1% Pro/Pro, 12% Pro/Arg, and 87% Arg/Arg. We found an association between the TGF-ß1 genotype encoding proline at codon 10 and renal dysfunction. At 7 years post-transplantation, 26% (23/89) of the patients with the heterozygous Pro/Leu or homozygous Pro/Pro genotype had renal dysfunction vs only 7% (3/42) of the patients with the homozygous Leu/Leu genotype (p=0.017). For the TGF-ß1 codon 25 genotypes, we found no association between TGF-ß1 genotypes and renal dysfunction.

Our data support the hypothesis that TGF-ß1 is involved in the process leading to renal insufficiency in cyclosporin treated cardiac allograft recipients. In these patients the presence of TGF-ß1 Pro^{10} might be a risk factor.

Introduction

Immunosuppressive therapy is vital for the prevention and treatment of allograft rejection in organ transplant recipients. After the introduction of the immunosuppressant cyclosporin A (CsA) in the late-seventies both the graft and patient survival remarkably increased. Unfortunately, chronic treatment with CsA showed to have important side effects. A high proportion of patients treated with CsA suffer from hypertension, hyperlipidemia, and renal insufficiency.[1] Moreover, CsA seems to produce a greater degree of dysfunction in native kidneys than in renal transplants.[2] The registry of the International Society for Heart and Lung Transplantation reported that 4 years after cardiac transplantation 24% of the allograft recipients had developed renal dysfunction.[1] This complication negatively affects patient survival. At our center, 1-year survival after starting of dialysis of cardiac allograft recipients was only 60%.[3] In that retrospective study, for patients who maintained stable, good renal function, CsA dose and trough levels were not different from those patients who needed renal replacement therapy. This may suggest that CsA nephrotoxicity can result from individually determined susceptibility to CsA. Renal insufficiency induced by CsA is characterized by interstitial fibrosis, tubular atrophy and glomerular vascular lesions.[4] Immunohistochemistry has shown that kidneys with these morphologic abnormalities express the cytokine transforming growth factor (TGF)-ß1.[5-7] TGF-ß1 is a multifunctional growth factor with profibrogenetic properties. CsA can stimulate the in vivo production of TGF-ß.[8, 9] In experimental chronic nephropathy an association was found between the characteristic histological abnormalities induced by CsA and increased expression of TGF-ß1 in the affected kidney.[2, 10] Thus, TGF-ß1 may mediate CsA-stimulated nephrotoxicity. Polymorphisms in the signal sequence genetically control the production capacity of TGF-ß1, i.e., a substitution at amino acid position 10 and 25, $Leu^{10}\rightarrow Pro$ and $Arg^{25}\rightarrow Pro$, respectively.[11-13] These TGF-ß1 gene polymorphisms have been associated with hypertension, atherosclerosis, myocardial infarction, and cardiomyopathy.[14-17]

To assess the importance of genetic polymorphisms within key genes involved in the pathogenesis of CsA-induced nephotoxicity, we investigated whether functional polymorphisms in the signal sequence of the TGF-ß1 gene, $Leu^{10}\rightarrow Pro$ and $Arg^{25}\rightarrow Pro$ are associated with a predisposition for kidney dysfunction of CsA treated cardiac allograft recipients.

Materials and methods

Subjects

The study population consisted of 178 sequential heart transplant recipients who survived at least 1 year with a minimal follow-up of 7 years (mean, 8.5±2.7 years). Peripheral blood cells were available for analysis from 168 patients (144 males and 24 females). Patients received transplants in Rotterdam between June 1984 and September 1992. The indications for heart transplantation were ischemic heart disease (n=85), dilated cardiomyopathy (n=75), and primary valvular disease (n=8). At the time of transplantation, all patients had a creatinine clearance of >30 ml/min. Ninety-four volunteers served as normal controls. Both the patient population and control group consisted mainly (>95%) of Caucasians. The TGF-ß1 genotypes were determined from DNA samples isolated from peripheral blood samples.

DNA isolation

We extracted genomic DNA from 10^6 peripheral blood mononuclear cells (PBMC), using the method described by Boom *et al.* al.[18] The vast majority of PBMC were collected before transplantation. PBMC were lysed in a buffer containing triton X-100 and guanidinium-iso-thiocyanate. The DNA was bound to celite, washed twice with a buffer containing guanidinium-iso-thiocyanate, twice with 70% ethanol and once with acetone. The pellet was vacuum dried and dissolved in 100 µl double-distillated H_2O. The DNA was eluted by incubation at 56°C for 10 min. After centrifugation, the supernatant, containing the genomic DNA, was removed and placed into a clean tube.

DNA amplification

The DNA was amplified using polymerase chain reaction (PCR) in a GeneAmp PCR system 9600 (PE Biosystems, Norwalk, CT, USA). We performed PCR with sequence specific primers for TGF-ß1 covering the first exon sense primer: 5' ACT GCG CCC TTC TCC CTG 3' and anti-sense primer: 5' CTT CAC CAG CTC CAT GTC GAT AG 3'. Two µl DNA was added to 28 µl PCR mixture containing 0.2 mM of each dATP, dTTP, dCTP, dGTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 M betaine monohydrate, 0.5 µM of each primer and 1 U AmpliTaq GOLD polymerase (PE Biosystems). After 10 minutes 94°C enzyme activation, samples were subjected to 40 cycles of respectively denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute. The last cycle was extended with 7 minutes at 72°C. To confirm amplification, the PCR

product was electrophoresed through a 2% agarose gel in 0.5X TBE, containing 0.2 ng/ml ethidium bromide.

Detection of TGF-ß1 polymorphisms by Sequence Specific Oligonucleotide Probing (SSOP)

We determined the studied TGF-ß1 polymorphisms (+869, Leu^{10}→Pro and +915, Arg^{25}→Pro) by dot blot hybridization as described by Perrey *et al.* al.[19] Probes for codon 10: 5' GCT GCT GCC GCT GCT GC 3' and 5' GCT GCT GCT GCT GCT GCT 3', and for codon 25: 5' GCC TGG CCG GCC GGC CG 3' and 5' GCC TGG CCC GCC GGC CG 3'. Two biotinilated oligonucleotide probes were used to determine each polymorphism. Two µl PCR product were spotted onto Hybond^{TM}-N+ membrane (Amersham Pharmacia, Buckinghamshire, UK) and treated with 0.5M NaOH and 1.5 M NaCl for 5 minutes to separate double-stranded amplified DNA, followed by a neutralization step with 1.5 M NaCl and 0.5 M Tris, pH 7.5 for 1 minute. We baked the membranes in a microwave for 5 minutes and immobilized DNA onto the membranes by crosslinking with UV for 1 minute. Thereafter blots were incubated in 10 ml of hybridization buffer containing 5X SSC, 0.5X Denharts solution, 0.2 M EDTA, 0.5% SDS and 0.1 ml sonicated herring sperm (Promega, Madison, Wisconsin, USA) at 42.5°C for 30 minutes. We added 400 ng of specific biotinilated oligonucleotide probe and allowed to hybridize for 90 minutes at 42.5°C. The membranes were washed twice with 5X SSC and 0.1% SDS at room temperature for 5 minutes, followed by stringency washing with 1X SSC and 0.1% SDS at 58°C (+869, Leu^{10}→Pro) and 61°C (+915, Arg^{25}→Pro) for 30 minutes. Before visualizing the hybridized probes, we washed the membranes in 0.15 M NaCl and 0.1 M Tris buffer pH 7.5 for 1 minute and treated with 0.5% blocking agent (Roche Diagnostics, Almere, The Netherlands) for 30 minutes. Subsequently, the membranes were incubated with a streptavidine horse radish labeled peroxidase conjugate, (Amersham Pharmacia) for 30 minutes at room temperature before detection by chemoluminiscence using the ECL system (Amersham Pharmacia). TGF-ß1 genotypes of controls and patients were determined in 2 replicate experiments.

Statistics

We used the chi square test to compare gender distribution and genotype frequencies between patients and controls. We used the Student's *t*-test to compare age. We analyzed the correlation CsA trough levels and serum creatinine levels using Spearman correlation-coefficient (*r*). We considered *p* values ≤0.05 significant.

Results

Clinical data

Table 1 shows the characteristics of the controls and the studied patient group. The mean age and gender distribution were significantly different between the controls and the cardiac allograft recipients ($p<0.01$). Renal dysfunction was defined as a serum creatinine ≥ 250 µmol/l. At 1 year 2% (3/168), at 3 year 7% (11/160), at 5 year 12% (18/152), and at 7 year 20% (26/131) of the patients had severe renal dysfunction. The increased serum creatinine was associated with decreased creatinine clearance (Table 1).

Table 1. Distribution of TGF-ß1 genotypes among normal controls and cardiac allograft recipients with serum creatinine <250 µmol/L and serum creatinine ≥250 µmol/L at 7 years after transplantation

	Controls	Heart transplant patients	
		serum creatinine < 250 µmol/l	serum creatinine ≥ 250 µmol/l
No of subjects	94	105	26
Age (± SD)	36.7 ± 10.3	44.0 ± 11.8*	47.9 ± 8.7*
Gender (M/F)	49/45	88/17	25/1
Underlying disease			
Ischemic heart disease		50	10
Dilated cardiomyopathy		51	13
Primary valvular disease		4	3
Creatinine clearance ml/min*		64 ± 30	23 ± 14
Genotype distribution			
Codon 10 Leu/Leu	37 (39%)	39 (37%)	3 (12%)
Leu/Pro	43 (46%)	62 (59%)	21 (81%)
Pro/Pro	14 (15%)	4 (4%)	2 (8%)
Codon 25 Arg/Arg	79 (84%)	91 (88%)	21(84%)
Arg/Pro	15 (16%)	12 (11%)	4 (16%)
Pro/Pro	0 (0%)	1 (1%)	0
Allele frequency			
Codon 10 Leu	62%	67%	52%
Pro	38%	33%	48%
Codon 25 Arg	92%	93%	92%
Pro	8%	7%	8%

*Mean ± SD.

At 1 year the serum creatinine levels were not correlated with the simultaneously determined CsA trough levels (r=0.001, p=0.68). These CsA trough levels ranged from 65 to 610 ng/ml. At 7 years post-transplantation we found no difference with respect to age, gender distribution and underlying disease between patients with renal dysfunction (n= 26) and those with normal renal function (n=131, Table 1). Of the 37 patients who survived less than 7 years, 25 had ischemic heart disease, 11 had dilated cardiomyopathy, and 1 had primary valvular disease as underlying disease. Of these 37 patients, 31 were male and 6 female, and their ages ranged from 21 to 63 years (mean 47.2 ± 10.2 year). Nine out of these 37 patients (24%) had severe renal dysfunction at the time of their deaths.

Renal function after heart transplantation in relation to TGF-ß1 gene polymorphisms

The differences in age and gender between the patients and controls did not affect the outcome of the TGF-ß1 genotype analysis because no association was found between age, gender, and TGF-ß1 codon 10 and codon 25 genotype distribution (p=0.61 and p=0.11; p=0.60 and p=0.22, respectively).

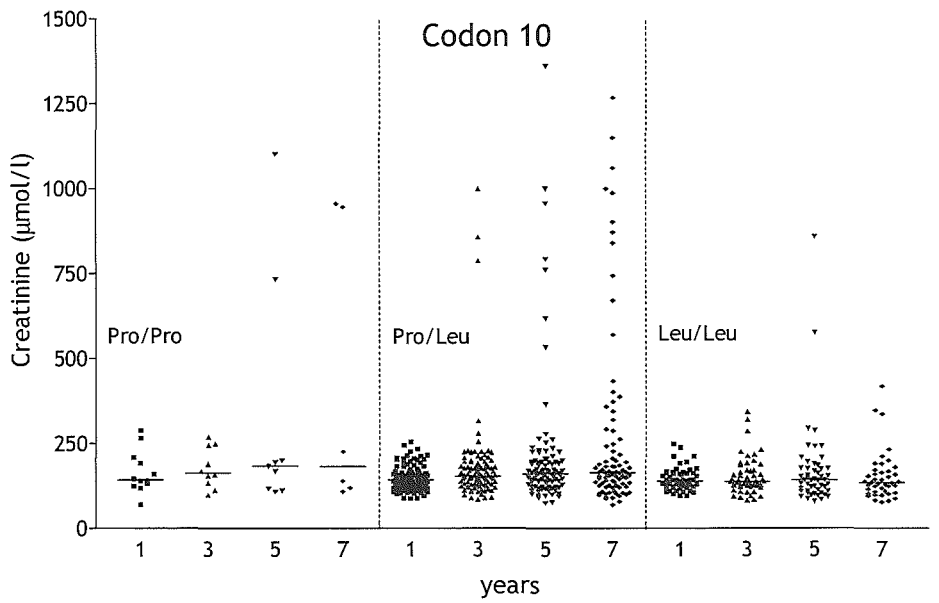

Figure 1. At 7 years post-transplantation significantly more cardiac allograft recipients with the TGF-ß1 codon 10 genotype Pro/Leu or Pro/Pro had renal dysfunction (serum creatinine ≥250 µmol/l) than did patients with the Leu/Leu genotype (23/89 vs 3/42, p=0.017).

We observed a significant difference observed in TGF-ß1 codon 10 genotype frequencies between the patients and controls. The TGF-ß1 codon 10 genotype distribution for the patients was 7% (12/168) homozygous for Pro/Pro, 61% (102/168) heterozygous for Pro/Leu, and 32% (54/168) homozygous for Leu/Leu. For the controls these percentages were 15% (14/94) Pro/Pro, 46% (43/94) Pro/Leu, and 39% (37/94) Leu/Leu (p=0.03). For TGF-ß1 codon 25 we saw no significant differences between the patients and controls. For the patients, the TGF-ß1 codon 25 genotype frequencies were 1% Pro/Pro, 12% Pro/Arg, and 87% Arg/Arg, respectively. Controls: 0% Pro/Pro, 16% Pro/Arg, and 84% Arg/Arg. After transplantation, the percentages for both TGF-ß1 codon 10 and codon 25 did not significantly change over time. We found comparable TGF-ß1 genotype distributions between patients who survived more than 7 years and those who did not.

We found an association between the TGF-ß1 genotype encoding Proline at codon 10 and renal dysfunction. At 7-years post-transplant, 26% (23/89) of the surviving patients with the heterozygous Leu/Pro or homozygous Pro/Pro genotype had renal dysfunction vs only 7% (3/42) of the patients with the homozygous Leu/Leu genotype (p=0.017, Figure 1).

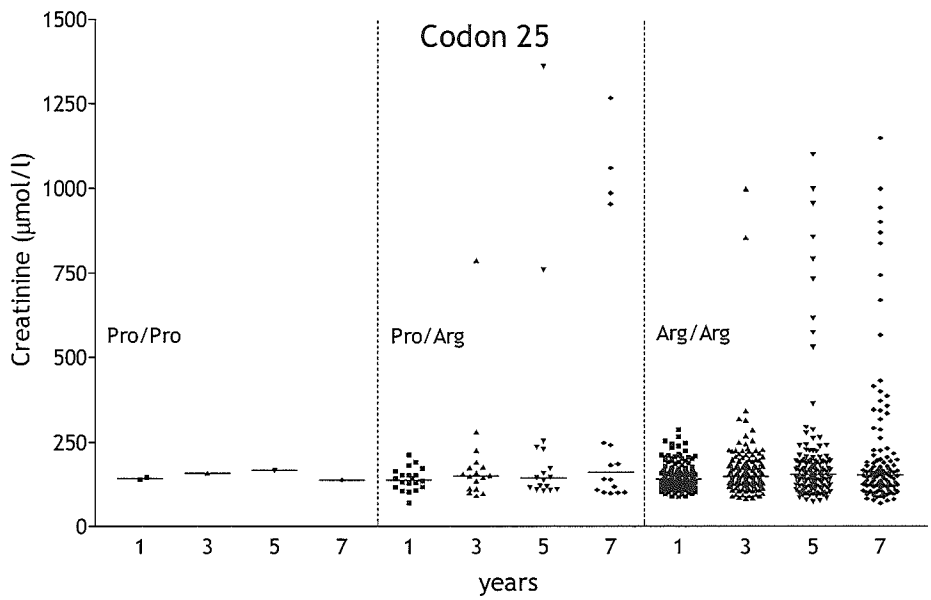

Figure 2. No association between TGF-ß1 codon 25 genotype and renal failure (serum creatinine ≥250 µmol/l) w as found.

The frequency of Pro^{10} allele was 48% in patients with renal dysfunction versus 33% in patients with normal renal function (Table 1). Of the patients who died within 7 years with renal dysfunction, the TGF-ß1 the Pro^{10} allele frequency was 44%. For the TGF-ß1 codon 25 genotypes, we found no association between TGF-ß1 genotypes and renal dysfunction (Table 1 and Figure 2).

Discussion

Several groups have reported that treatment with the immunosuppressant CsA is associated with increased TGF-ß1 production in vivo.[8, 9] This effect of CsA may be responsible for the nephropathy seen in non-renal allograft recipients who receive this agent as maintenance immunosuppressive therapy. At our center, renal dysfunction (serum creatinine levels ≥ 250 µmol/l) was seen in 20% (26/131) of CsA treated cardiac allograft recipients at 7 years post-transplantation (Table 1). This decline in renal function was associated neither with age, gender nor underlying cardiac disease of these patients. In addition, we found no correlation between CsA trough levels and serum creatinine levels at 1 year.

Two pathophysiologic mechanisms have been postulated by which CsA may exert its nephrotoxic effect. First, a vasomotor component of afferent arteriolar constriction that reduces the glomerular perfusion. Indeed, it seems that the intact sympathetic innervation of native kidneys in heart transplant recipients makes them more vulnerable to the stimulatory effect of CsA than denervated transplant kidneys. The second factor is a structural one characterized by replacement of vascular smooth muscle cells with hyaline material and the induction of interstitial fibrosis.[20] In both mechanisms the multifunctional cytokine TGF-ß1 may be involved. It stimulates the local renin-angiotesin system probably through endothelin release, resulting in increased renal vascular resistance and glomerular hypertension.[21] TGF-ß1 is also the key cytokine in the development of fibrosis and glomerulosclerosis.[5]

We have analyzed whether two polymorphisms in the signaling sequence of the TGF-ß1 gene, i.e., a substitution at amino acid position 10 ($Leu^{10}\rightarrow Pro$) and at position 25 ($Arg^{25}\rightarrow Pro$), are associated with increased susceptibility to CsA-induced nephrotoxicity. We studied this in a patient population who survived at least 1 year. Although we can not exclude the possibility of patient selection, the TGF-ß genotypes of the patient group who survived less than 1 year (n=13) were comparable to the TGF-ß1 genotypes of the study population (n=168, data not shown). Analysis of the TGF-ß1 genotypes for codon 10 ($Leu^{10}\rightarrow Pro$) revealed that

renal dysfunction was predominantly found in patients with either the heterozygous Leu/Pro genotype or the homozygous Pro/Pro genotype at 7 years post-transplantation (Figure 1). In the patient population (n=9) who died within 7 years with renal dysfunction, the TGF-ß1 codon 10 allele distribution was comparable to that in patients with serum creatinine ≥ 250 µmol/l at 7 years, 44% and 48% respectively. Therefore, our TGF-ß1 codon 10 ($Leu^{10}\rightarrow Pro$) gene polymorphism findings suggest that patients with the Pro allele are more susceptible for CsA induced nephrotoxicity (Table 1). TGF-ß1 codon 25 ($Arg^{25}\rightarrow Pro$) gene polymorphism was not associated with CsA-induced renal dysfunction, which is line with findings by the group of Hutchinson.[22] Gene polymorphism studies do not provide information on actual protein production but show an association with a complex disease. Currently, it is not clear whether a Leucine or Proline at amino acid position 10 of the signaling sequence of the TGF-ß1 gene results in high active TGF-ß1 concentrations in vivo. Awad *et al.* al. reported that patients with cystic fibrosis with a Leucine residue at codon 10 have elevated circulating levels of TGF-ß1, while Yamada et al. showed that Japanese women with osteoporosis who have a Proline residue at this position have the highest TGF-ß1 serum levels.[11, 12]
From our studies, we concluded that renal insufficiency in cyclosporin treated cardiac allograft recipients may be associated with the Proline allele at amino acid position 10 of the signaling sequence of the TGF-ß1 gene.

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Chapter 8

Intragraft Heme Oxygenase-1 and coronary artery disease after heart transplantation

Cécile Holweg
Aggie Balk
Jasper Snaathorst
Sandra van den Engel
André Uitterlinden
Bert Niesters
Lex Maat
Pieter Zondervan
Willem Weimar
Carla Baan

Submitted

Abstract

We investigated the role and mechanism of HO-1 in the development of Transplant Coronary Artery Disease. Peri-operative tissue injury triggers the development of Transplant Coronary Artery Disease (TCAD). Animal studies showed that induction of heme oxygenase (HO)-1 protects the donor organ from TCAD.

Intragraft mRNA expression levels of HO-1, HIF-1α, TGF-ß, FLIP, and the Bcl-2/Bax balance were measured in myocardial biopsies taken at the end of the transplantation procedure, at one week and 10 months after transplantation. Immunohistochemical staining of HO-1 was performed to determine its origin. To assess possible predisposition for development of TCAD, the promoter $(GT)_n$ polymorphism was studied.

At time zero, no differences in mRNA expression for any of the measured parameters were found between TCAD positive and negative patients. At one week, expression of HO-1 and TGF-ß was higher in grafts that developed TCAD ($p=0.001$ and $p=0.0002$). HO-1 was mainly produced by macrophages. These higher levels were accompanied by a pro-apoptotic shift in Bcl-2/Bax ($p=0.02$), suggesting proneness for apoptosis via the mitochondrial pathway. At 10 months, again HO-1 and TGF-ß levels were high in TCAD positive patients ($p=0.02$ and $p=0.05$). Expression of apoptotic markers was comparable. No predisposition for TCAD was found when we studied the $(GT)_n$ polymorphism.

HO-1 expression is associated with coronary artery disease in human cardiac allograft recipients. Our results suggest that upregulation of HO-1 is an adaptive response to tissue injury and inflammation, reflecting damage due to the transplantation procedure. This upregulation is not sufficient to protect against TCAD.

Introduction

Transplant coronary artery disease (TCAD) is the cause of morbidity and mortality in a considerable number of heart transplant recipients.[1] TCAD is a chronic inflammatory process characterized by progressive and diffuse intimal thickening, due to migration of macrophages, migration and proliferation of T-cells and smooth muscle cells, collagen accumulation and fibrosis.[2] Oxidative stress due to peri-operative ischemia followed by reperfusion might be an early trigger for TCAD.[2, 3] Oxidative stress leads to apoptosis and necrosis of endothelial cells, vascular smooth muscle cells and cardiac myocytes. This could subsequently result in activation of cytokines and growth factors, like IFN-γ, PDGF-α, bFGF and TGF-ß, that are involved in processes causing intimal thickening of the coronary arteries of human cardiac allografts.[4, 5]

The anti-oxidant enzyme heme oxygenase (HO)-1 catalyzes the degradation of heme into biliverdin, iron and carbon monoxide (CO). Biliverdin is subsequently converted to bilirubin. These catabolic end products are thought to be responsible for the anti-inflammatory, anti-oxidant, and anti-apoptotic properties of HO-1.[6] Upregulation of HO-1 might be a mechanism to protect cells from damage due to ischemia, reperfusion and inflammation.[6, 7] Consequently HO-1 might prevent the induction of TCAD. Indeed, animal studies demonstrated that induction of HO-1 with an adenovirus-HO-1 construct or by cobalt protoporphyrin (CoPP) prior to or shortly after transplantation protects against the development of TCAD.[8, 9]

To investigate whether HO-1 represents a factor by which the donor organ is protected from the initiation and progression of TCAD in human cardiac recipients, we measured intragraft mRNA expression levels of HO-1. To study the mechanism by which HO-1 acts, we determined Hypoxia Inducible Factor-(HIF)1α, a transcription factor for HO-1; TGF-ß, a growth factor known to be involved in the development of TCAD[4, 10] and a regulator of HO-1 production[11]; FLIP (short and long), an anti-apoptotic marker of the TNF-α–Fas/FasL induced apoptosis route and Bax and Bcl-2, pro- and anti-apoptotic markers of the mitochondrial apoptosis pathway. These parameters were measured in myocardial biopsies (MB) taken at the time of graft implantation (time zero), early after transplantation (one week) and to the end of the first year after transplantation (10 months). In addition, we measured HO-1 protein by immunohistochemistry to study the origin of HO-1. Finally, we studied a $(GT)_n$ repeat polymorphism in the promoter region of the HO-1 gene in patients and their accompanying donors, to identify a possible predisposition for the development of TCAD. This $(GT)_n$ repeat affects HO-1 promoter activity, whereby the number of repeats is inversely related to the

activity.[12, 13] We hypothesize that, heart transplant recipients with less repeats or receiving grafts from donors with less repeats, might be protected from development of TCAD.

Material and methods

Patients

Intragraft mRNA expression of HO-1, HIF-1α, TGF-ß, $FLIP_{S+L}$, Bax, Bcl-2 and 18S RNA (housekeeping gene) was measured in myocardial biopsies (MB) from heart transplant recipients with and without TCAD. TCAD was diagnosed at one-year after transplantation by visual assessment of the coronary angiogram and defined as all abnormalities of the epicardial as well as the intramyocardial branches, including minimal wall irregularities. Coronary arteriograms had been assessed long before mRNA measurements and genotyping. An angiographic diagnosis was made by consensus of two observers with experience in the evaluation of post-transplant arteriograms. Pre-transplant arteriograms of the donor were not available.[14]

MB were taken from the left ventricle at the end of graft implantation, before weaning from extra corporeal circulation (time zero, without TCAD, n=12 and with TCAD, n=11), and from the right side of the interventricular septum early after transplantation (week one, without TCAD, n=8 and with TCAD, n=8) and during the development of TCAD (10 months after transplantation, without TCAD, n=13 and with TCAD, n=14). All biopsies were free of signs for acute rejection (grade 0 or 1A, ISHLT criteria).[15] Unfortunately, the studied biopsies were not all from a consistent cohort of patient. Biopsies of all three time points were available from seven patients and of two time points from 10 patients (Figure 2). Demographics of patients at the different time points of mRNA measurements are summarized in table 1. All patients were on Cyclosporin A and low dose steroids as maintenance immunosuppressive therapy. Cyclosporin A was aimed to keep 12 h through levels between 250 and 350 ng/ml in the first six months after transplantation and between 100 and 200 ng/ml after six months. Cytomegalovirus (CMV) infection was defined as any appearance of immunoglobulin M or isolation of CMV from urine, throat or blood or any demonstration of immediate early antigen. CMV disease was diagnosed when infection co-existed with two of the following symptoms: fever of more than 38°C for at least two consecutive days, gastrointestinal, lung, retina or central nervous system involvement, leucopenia, thrombocytopenia, elevation of serum alanine or aspartate aminotransferases. CMV seronegative recipients received seronegative blood products and were, when receiving a heart of a

Table 1. Characteristics of patients with and without TCAD at time points of intragraft mRNA expression measurement

	Before transplantation			Week one			Month 10		
	TCAD neg n=12	TCAD pos n=11	p-value	TCAD neg n=8	TCAD pos n=8	p-value	TCAD neg n=13	TCAD pos n=14	p-value
Recipient age (years)*	54.0 (19.2-65.2)	57.3 (14.2-65.3)	0.83**	54.0 (19.2-61.9)	56.3 (41.9-61.3)	0.80**	52.2 (19.2-65.0)	55.2 (14.2-63.7)	0.56**
Donor age (years)*	31.5 (13.0-52.0)	38.0 (17.0-49.0)	0.21**	32.5 (13.0-43.0)	40.5 (31.0-49.0)	0.07**	34.0 (22.0-52.0)	36.0 (17.0-49.0)	0.96**
Recipient gender (M/F)	11/1	9/2	0.59‡	7/1	7/1	1.0‡	13/0	12/2	0.48‡
Donor gender (M/F)	7/5	5/6	0.68‡	7/1	4/4	0.28‡	7/6	5/9	0.45‡
Gender mismatch (no/yes)	6/6	7/4	0.68‡	6/2	5/3	1.0‡	7/6	7/7	1.0‡
Cause of braindeath (donor)									
Cerebrovascular/trauma/unknown	7/4/1	6/5/0	0.68‡	4/3/1	5/3/0	1.0‡	7/5/1	6/7/1	0.84‡
Time of brain death of donor (min)*	619 (387-920)	538 (167-810)	0.19**	598 (387-920)	529 (390-810)	0.78**	595 (390-920)	535 (217-810)	0.12**
Vasoactive drugs in donor									
(no/yes/unknown)	2/9/1	0/11/0	0.34‡	2/6/0	0/8/0	0.47‡	1/11/1	0/12/2	1.0‡
Heart disease (recipient)									
CMP/IHD/other	4/7/1	2/8/1	0.80‡	5/3/0	1/6/1	0.12‡	4/8/1	4/8/1	1.0‡
Cold ischemia time (min)*	171 (136-197)	190 (127-220)	0.37**	173 (140-197)	178 (140-279)	0.54**	171 (110-245)	178 (128-279)	0.46**
HLA AB mismatches*	3.0 (2.0-4.0)	3.0 (2.0-4.0)	0.15**	3.0 (2.0-4.0)	4.0 (2.0-4.0)	0.50**	3.0 (2.0-4.0)	3.0 (1.0-4.0)	0.33**
HLA-DR mismatches*	2.0 (0.0-2.0)	2.0 (0.0-2.0)	0.78**	2.0 (0.0-2.0)	2.0 (0.0-2.0)	0.72**	2.0 (0.0-2.0)	2.0 (0.0-2.0)	0.55**
Induction therapy									
None/anti-T-cell/anti-CD25	1/7/4	2/5/4	0.86‡	0/5/3	1/5/2	1.0‡	3/9/1	3/8/3	0.75‡
Nr of AR first year*	1.0 (0.0-4.0)	4.0 (0.0-5.0)	0.04**	1.0 (0.0-3.0)	1.5 (0.0-5.0)	0.57**	1.0 (0.0-3.0)	2.0 (0.0-5.0)	0.33**
CMV infection (no/yes)	9/3	5/6	0.21‡	7/1	4/4	0.28‡	8/5	8/6	1.0‡
CMV disease (no/yes)	10/2	9/2	1.0‡	8/0	6/2	0.47‡	9/4	9/5	1.0‡
Total cholesterol at 1-year (nmol/L)*	7.0 (4.0-9.9)	6.5 (4.3-11.0)	0.73**	5.1 (4.0-9.1)	7.4 (4.7-11.0)	0.23**	6.4 (4.8-9.1)	6.4 (4.7-11.0)	0.98**
Triglycerides at 1-year (nmol/L)*	2.1 (0.7-4.4)	2.9 (1.3-4.8)	0.48**	2.0 (0.7-2.4)	2.6 (0.9-3.2)	0.34**	2.8 (1.2-5.8)	2.1 (0.9-3.2)	0.10**
HMG-CoA-reductase inhibitors (no/yes)	10/2	11/0	0.48‡	6/2	8/0	0.47‡	9/4	11/3	0.68‡

AR: acute rejection, CMP: dilated cardiomyopathy, IHD: ischemic heart disease. Time of brain death of the donor: from establishment of brain death to the harvesting of the heart. * Median with range between parentheses. P-values are obtained by ** Mann Whitney and ‡ (exact) χ^2-test.

seropositive donor, treated with anti-CMV hyperimmunoglobulins for passive immunization.

For analysis of the $(GT)_n$ repeat polymorphism in the HO-1 gene, we genotyped DNA of 292 out of 329 consecutive heart transplant recipients and 243 of their donors. This cohort contains also the patients of whom intragraft mRNA was measured. Patients were transplanted in Rotterdam between June 1984 and January 1998. At one-year, 85 recipients were diagnosed positive and 207 patients negative for TCAD according to their first year coronary angiogram. Because we did not have DNA of all donors, we analyzed 74 donors of the recipients with TCAD and 169 donors of recipients without TCAD. To study possible associations between the repeat polymorphism and accelerated TCAD, alleles were grouped according to Yamada et al. into 3 classes, short alleles (S: <27 repeats), medium alleles (M: 27–32 repeats) and long alleles (L: >32 repeats).[12] Characteristics of the genotyped recipients are summarized in table 2.

All heart transplant recipients gave permission to use their material and data for research purposes.

Table 2. Characteristics of genotyped cardiac recipients without or with transplant coronary artery disease (TCAD)

	TCAD neg	TCAD pos	p-value
No.	207	85	
Recipient age (years)*	46.5±11.7	49.4±10.7	0.05†
Donor age (years)*	25.3±8.8	30.±9.6	<0.0001**
Recipient gender (M/F)	175/32	71/14	0.86‡
Donor gender (M/F)	126/81	52/33	1.0‡
Gender mismatch (no/yes)	126/81	52/33	1.0‡
Original heart disease			
CMP/IHD/other	92/106/9	28/51/5	0.21‡
Cold ischemia time (min)*	165±38	171±41	0.27**
HLA-AB mismatches*	3.0±0.9	2.9±0.9	0.65†
HLA-DR mismatches*	1.4±0.6	1.5±0.6	0.23†
Induction therapy			
None/ATG/OKT3/anti-CD25	57/53/70/27	19/26/22/18	0.17‡
No of AR in the first year*	1.7±1.5	2.0±1.7	0.25†
CMV infection (no/yes)	114/93	47/38	1.0‡
CMV disease (no/yes)	172/35	71/14	1.0‡
Total cholesterol (nmol/L)*	7.1±1.7	7.6±2.3	0.04**
Triglycerides (nmol/L)*	2.4±1.1	2.5±1.3	0.71†
HMG-CoA-reductase inhibitors (no/yes)	190/70	75/10	0.0046‡

CMP: dilated cardiomyopathy, IHD: ischemic heart disease, AR: acute rejection.* Mean ± SD. p-values were obtained by **Student's t-test, †Mann-Whitney-test and ‡(exact) χ^2-test.

Total RNA extraction, cDNA synthesis and real-time polymerase chain reaction

MB were snap-frozen in liquid nitrogen and stored at -80°C until use. Total RNA isolation and cDNA synthesis were performed as described previously.[5] The mRNA levels of HO-1, HIF-1α, TGF-ß, FLIP, Bcl-2 and Bax and the RNA level of 18S were measured using real-time PCR in the ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described in detail before.[16] For TGF-ß and 18S, pre-developed TaqMan® assays (Applied Biosystems) and for Bax and Bcl-2 primers and FRET probes (Biosource International, Inc., Camarillo, CA, USA) were used. For HO-1, HIF-1α and FLIP (detecting both $FLIP_S$ and $FLIP_L$) we designed the following primers and probes: HO-1 sense primer: 5'-TGC-TCA-ACA-TCC-AGC-TCT-TTG-A-3'; HO-1 anti-sense primer: 5'-GCA-GAA-TCT-TGC-ACT-TTG-TTG-CT-3'; HO-1 probe: FAM-5'-AGT-TGC-AGG-AGC-TGC-TGA-CCC-ATG-AC-3'; HIF-1α sense primer: 5'-AAC-ATG-ATG-GTT-CAC-TTT-TTC-AAG-C-3'; HIF-1α anti-sense primer: 5'-GTC-AGC-TGT-GGT-AAT-CCA-CTT-TCA-T-3'; HIF-1α probe: FAM-5'-TAG-GAA-TTG-GAA-CAT-TAT-TAC-AGC-AGC-CAG-ACG-3'; FLIP sense primer: 5'-AGG-CAA-GAT-AAG-CAA-GGA-GAA-GAG-T-3'; FLIP anti-sense primer: 5'-TTT-TCT-ATT-AAA-TCC-AGT-TGA-TCT-GGG-3'; FLIP probe: FAM-5'-TCT-TGG-ACC-TTG-TGG-TTG-AGT-TGG-AGA-AA-3'. Optimal annealing and extension temperatures were: 59°C for HO-1, HIF-1α and $FLIP_{(S+L)}$ and 60°C for TGF-β, Bcl-2, Bax, and 18S. Standard curves with serial dilutions of known amounts of the target molecules were used to determine the mRNA concentrations in MB. The measured mRNA concentrations for the different molecules were standardized for the 18S concentration.

Immunohistochemical analysis

Double staining of HO-1 with CD3 (T-cells), CD68 (macrophages) and CD31 (endothelial cells) was performed on snap frozen MB specimens (n=12, taken at week one), cut in 5 μm sections, air-dried and fixed in acetone. The antibodies used to stain the samples were rabbit-anti-human (HO-1: clone H-105, Santa Cruz, Biotechnology Inc, Santa Cruz, CA) and mouse-anti-human (CD3: clone SK7, Becton & Dickinson, San Jose, CA; CD68: clone KP1 and CD31: clone JC/70A Dako, Glostrup, Denmark). For the detection of HO-1, we used the ABC method and the PAP method for CD3, CD68 and CD31. In brief, slides were rinsed in phosphate-buffered saline and incubated with rabbit-anti-human or mouse anti-human antibodies. The slides were then rinsed and incubated with biotinylated goat-anti-rabbit antibody (HO-1; Dako) or rabbit-anti-mouse IgG (CD3, CD68 and CD31; Biogenex, San Ramon CA). Again the slides were rinsed and subsequently labeled with the ABC complex or PAP complex. The enzyme was detected with Fast Blue

BB Salt (HO-1; Sigma-Aldrich chemie BV, Zwijndrecht, The Netherlands) or DAB (CD3, CD68 and CD31; Sigma) as a substrate, following the manufacturers instructions. In control sections, the primary antibody was omitted.

DNA isolation and genotyping of the $(GT)_n$ repeat polymorphism in the HO-1 promoter

Genomic DNA isolation and genotyping of the repeat polymorphism in the promoter region of the HO-1 gene was performed as previously described.[17] For PCR, we used a 5' FAM labeled forward primer (5'-FAM-AGA-GCC-TGC-AGC-TTC-TCA-GA-3') and a reverse primer (5'-ACA-AAG-TCT-GGC-CAT-AGG-AC-3') covering the GT repeat. The allele sizes observed were confirmed by sequencing DNA of homozygous patients.

Statistics

Characteristics of patients with and without TCAD were compared using the (exact) χ^2-test for discrete variables and Mann Whitney or Student's t-test for continues variables, as appropriate. When the exact χ^2-test was used, the exact mid p-value was calculated, using StatXact software (CYTEL Software corporation, Cambridge, MA, USA).

To compare mRNA expression levels between patients with and without TCAD at the different time points, the Mann Whitney test was used. For analysis of the HO-1 $(GT)_n$ polymorphism, we grouped the alleles in short (S, <27), medium (M, 27-32) and long (L, >32) alleles, according to Yamada et al.[12] The χ^2-test was used to compare HO-1 $(GT)_n$ repeat polymorphism allele and genotype frequencies. For all tests, $P \leq 0.05$ was considered significant.

Results

Patient demographics

The characteristics of recipients with and without TCAD were comparable at all time points of mRNA measurements, except for the number of acute rejections at time zero (Table 1). This was higher in the recipients in which TCAD was diagnosed at one year. For the genotyped patient group, recipient and donor age were higher and the use of HMG-CoA-reductase inhibitors was lower in the patients positive for TCAD compared to recipients without TCAD. All other characteristics were comparable between the two groups (Table 2).

Intragraft mRNA expression

No differences in mRNA expression levels of any of the measured parameters were found between patients without (n=12) and with (n=11) accelerated TCAD in the time zero biopsies (Table 3). At one week, however, higher mRNA expression levels for HO-1 and TGF-ß in biopsies from patients with TCAD compared to grafts of patients without TCAD were found (Table 3 and Figure 1A). In addition, we found a lower ratio of the apoptotic markers Bcl-2/Bax in patients with signs of TCAD (Table 3, Figure 1B). The HIF-1α mRNA expression levels were comparable between the patient groups (Table 3) and also no differences for FLIP between the two patient groups were measured (Table 3, Figure 1B). In biopsies taken at 10 months after transplantation, again, higher HO-1 and TGF-ß mRNA expression levels were measured in patients with a positive coronary angiogram (Table 3, Figure 1C). In these samples, no disbalance in apoptotic markers was detected between patients with or without TCAD (Table 3, Figure 1D) and comparable HIF-1α expression was found (Table 3).

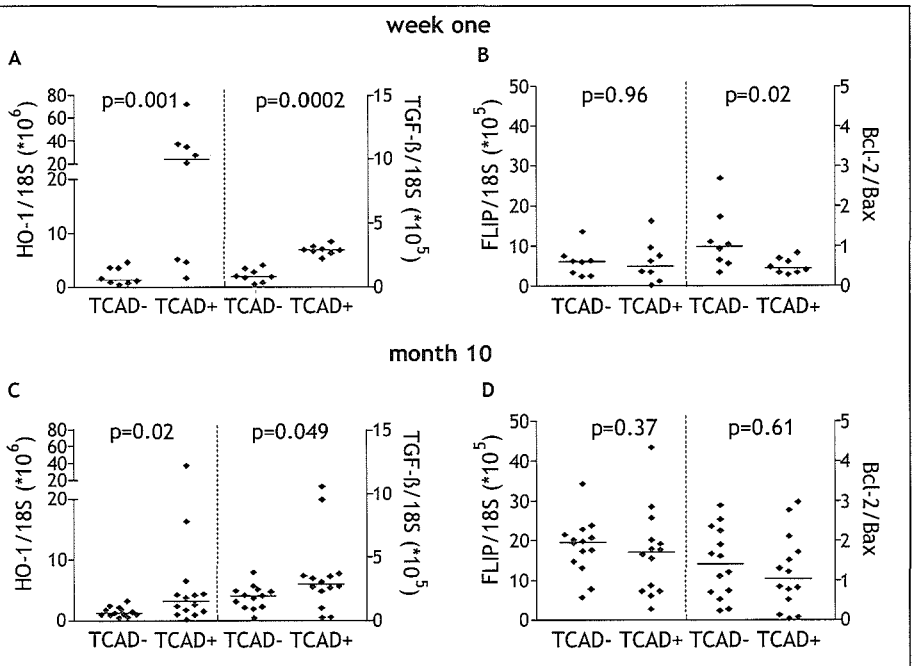

Figure 1. Intragraft mRNA expression at week one and month 10 after transplantation of Heme-Oxygenase-1 and TGF-ß (A and C) and apoptotic markers FLIP and Bcl-2/Bax ratio (B and D) in patients with and without transplant coronary artery disease (TCAD). A significant higher mRNA expression for HO-1 and TGF-ß accompanied by a pro-apoptotic shift in the Bcl-2/Bax balance at one week after transplantation was found in patients with TCAD. The higher HO-1 and TGF-ß levels were again found at 10 months in TCAD positive patients.

In figure 2, we show the HO-1 mRNA expression over time, with a connecting line between biopsies of one patient. The consistent cohort of patients is too small for statistical analysis, but we observed that in TCAD positive patients the HO-1 mRNA expression increases at week one and decreases thereafter, which we did not observe in TCAD negative cardiac recipients.

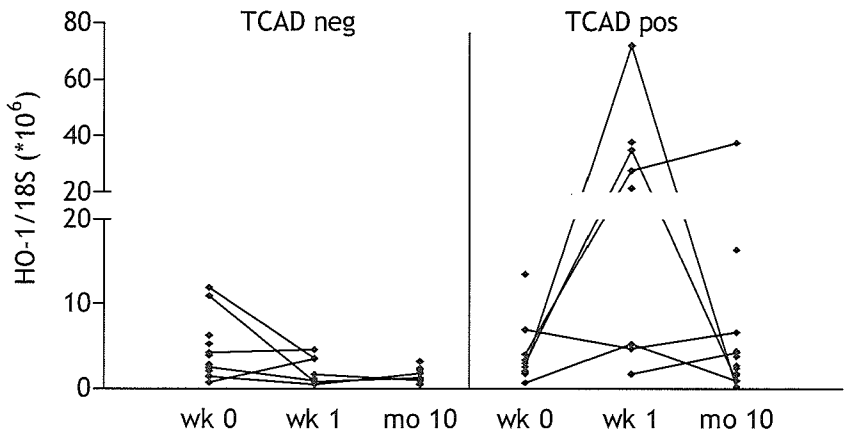

Figure 2. Intragraft Heme Oxygenase-1 mRNA expression in patients with and without TCAD over time. When more than one biopsy was available from a patient, these are connected with a line. Wk 0: time point at time of transplantation, wk 1: one week after transplantation, mo 10: 10 months after transplantation.

Immunohistochemical analysis

To determine the origin of the HO-1 production, we studied week one biopsies, because the differences in mRNA expression between patients with and without TCAD was most striking at this time point. Few CD3 (T-cells) positive cells were present in the biopsies. These CD3 cells did not express HO-1. Of the CD31 (endothelial cells) positive cells, a few cells were positive for HO-1 (<10%). HO-1 expression was mainly seen in CD68 (macrophages) positive cells. All biopsies (n=12) contained CD68 positive cells (range 11-100 cells), which was in accordance with previous findings.[18] In 8 of these biopsies, 50-90% of the macrophages expressed HO-1. No double staining for HO-1 and cardiomyocytes was performed, but morphological analysis of the biopsies showed no HO-1 positive cardiomyocytes.

Table 3. Intragraft mRNA expression of HIF-1α, HO-1, TGF-ß, $FLIP_{S+L}$ and the Bcl-2/Bax balance in myocardial biopsies of patients with and without TCAD at one year

	Time zero		p-value	Week 1		p-value	Month 10		p-value
	TCAD– (n=12)	TCAD+ (n=11)		TCAD– (n=8)	TCAD+ (n=8)		TCAD– (n=13)	TCAD+ (n=14)	
HIF-1α	0.8 (0.04-1.99)	0.5 (0.23-1.55)	0.31	0.4 (0.05-1.06)	0.3 (0.05-0.59)	0.38	0.6 (0.08-0.91)	0.6 (0.05-1.51)	0.25
HO-1	3.3 (0.80-11.8)	2.5 (0.70-13.4)	0.52	1.4 (0.50-4.60)	24.6 (1.7-72.2)	**0.001**	1.3 (0.50-3.20)	3.2 (0.20-37.7)	**0.02**
TGF-ß	1.3 (0.02-5.00)	1.0 (0.60-4.80)	0.37	0.8 (0.20-1.70)	2.9 (2.20-3.50)	**0.0002**	2.0 (0.50-3.20)	2.9 (0.20-10.6)	**0.05**
$FLIP_{S+L}$	6.9 (4.20-10.9)	4.3 (1.40-12.8)	0.12	6.2 (2.40-13.6)	5.0 (0.20-16.3)	0.96	19.8 (5.60-34.4)	17.1 (2.70-43.4)	0.37
Bcl-2/Bax	1.7 (0.20-4.20)	1.1 (0.30-3.60)	0.36	1.0 (0.40-2.70)	0.5 (0.30-0.80)	**0.02**	1.2 (0.20-2.90)	1.0 (0.03-3.00)	0.61

Numbers are the median of the mRNA expression normalized for 18S expression with the range between parentheses. For HO-1 the ratios are multiplied by 10^6 and for HIF-1α, TGF-ß and FLIP by 10^5. P-values are obtained by Mann Whitney test.

Table 4. Allele and genotype frequencies of the $(GT)_n$ repeat polymorphism in the promoter of the HO-1 gene

	Patients	Donors	Patients		Donors	
	Total	Total	GVD–	GVD+	GVD–	GVD+
Alleles	n=584	n=486	n=414	n=170	n=338	n=148
S	200 (0.34)	174 (0.36)	147 (0.36)	53 (0.31)	122 (0.36)	52 (0.35)
M	349 (0.60)	276 (0.57)	241 (0.58)	108 (0.64)	193 (0.57)	83 (0.56)
L	35 (0.06)	36 (0.07)	26 (0.06)	9 (0.05)	23 (0.07)	13 (0.09)
Genotypes	n=292	n=243	n=207	n=85	n=169	n=74
S/S	41 (14,0%)	28 (11.5%)	31 (15.0%)	10 (11.8%)	20 (11.8%)	8 (10.8%)
S/M	107 (36.7%)	107 (44.0%)	77 (37.2%)	30 (35.3%)	74 (43.8%)	33 (44.6%)
S/L	11 (3.8%)	11 (4.5%)	8 (3.9%)	3 (3.5%)	8 (4.7%)	3 (4.1%)
M/M	111 (38.0%)	75 (31.0%)	74 (35.7%)	37 (43.5%)	52 (30.8%)	23 (31.1%)
M/L	20 (6.8%)	19 (7.8%)	16 (7.7%)	4 (4.7%)	15 (8.9%)	4 (5.4%)
L/L	2 (0.7%)	3 (1.2%)	1 (0.5%)	1 (1.2%)	0 (0%)	3 (4.1%)

No significant differences were found for allele or genotype frequencies between patients and donors or between patients or donors with or without TCAD.

Repeat polymorphism $(GT)_n$ in the promoter of the HO-1 gene

The number of repeats ranged from 12 to 40, whereby alleles 23 and 30 were most common in our population of 292 heart transplant recipients. When we compared allele and genotype frequencies of the patient group with the donors, no significant differences were found (Table 4). Dividing the patient group into patients without and with TCAD, again resulted in comparable allele and genotype frequencies (Table 4). Also no differences were found when we analyzed the allele and genotype frequencies of the donor in relation to the development of TCAD in the transplanted heart (Table 4). We also analyzed the $(GT)_n$ repeat polymorphism in relation to the development of TCAD using other allele categories, but this neither resulted in an association.

Discussion

We investigated HO-1 expression and the mechanism by which HO-1 acts in the initiation and progression of accelerated TCAD. We measured higher HO-1 and TGF-ß mRNA expression levels in biopsies taken from recipients with TCAD compared to patients without TCAD at both one week and at 10 months after transplantation. In the week one biopsies, these higher expression levels were accompanied by a shifted Bcl-2/Bax balance towards the pro-apoptotic marker Bax of the mitochondrial apoptosis pathway. This indicates that these patients might be prone to apoptosis and tissue injury due to apoptosis via this route.

The higher HO-1 mRNA expression levels in patients who developed TCAD within the first year was unexpected. In accordance with the findings in rodents, we presumed that HO-1 would have been higher in TCAD negative heart transplant recipients.[8, 9] Apparently, despite upregulation of HO-1, TCAD developed in our patients. However, in the animal studies, production of HO-1 was induced in the donor organ before transplantation with CoPP or an adenovirus construct containing HO-1. In our study, there was no artificial upregulation of HO-1 in the donor heart. In the biopsies of our heart transplant recipients, HO-1 induction could have been caused by cellular stress, such as hypoxia, ischemia/reperfusion and inflammation.[18, 19] Hypoxia activates the transcription factors HIF-1α, NFκB and AP-1 which subsequently lead to the upregulation of HO-1.[20] The higher HO-1 expression was not associated with upregulation of HIF-1α, showing that this transcription factor is not the initiator of the HO-1 production in our patient group. Reperfusion after a period of ischemia might also lead to upregulation of HO-1. During reperfusion, an oxygen burst occurs, whereby reactive oxygen species (ROS)

are formed.[21] ROS activate transcription factors, which then induce expression of pro-inflammatory cytokines leading to inflammatory responses.[22] HO-1 is able to suppress inflammatory reactions.[19] Indeed, our immunohistochemical results show that most of the HO-1 protein is produced by the infiltrating macrophages. This indicates that HO-1 is upregulated as an adaptive response and probably plays a role in the resolution of the inflammatory responses. ROS can also cause tissue damage by inducing mitochondrial membrane changes, leading to the release of apoptogenic factors, which is prevented by Bcl-2. Binding of Bax to Bcl-2 abolishes this protective effect.[21] Thus, the balance between Bcl-2 and Bax can reflect tissue injury due to apoptosis and the found the pro-apoptotic shift in the one week biopsies, suggests more tissue damage in patients who were going to develop TCAD.

We also confirmed earlier findings of higher TGF-ß mRNA expression in biopsies from patients with early signs of TCAD.[5] Since it is known that TGF-ß is involved in wound repair, we assume that TGF-ß is upregulated to repair damaged tissue due to peri-operative processes. Upregulation of HO-1 by TGF-ß might be a mechanism to govern the repair process.[11]

These considerations only explain the upregulation of HO-1 as an adaptive inflammatory response to avoid or limit oxidative damage. However, it does not explain why there might be more damage in some donor hearts compared to others. Therefore, we compared donor factors that might contribute to graft injury, like time to and cause of brain death of the donor or use of vasoactive drugs in the donor. This did also not clarify the question, as we could not find a relation between these parameters and development of TCAD.

It is also possible that the higher HO-1 expression in our TCAD positive group is not just the result of an adaptive response to tissue damage or inflammation, but of a genetically predisposed capacity to produce higher amounts of HO-1. To investigate this, we analyzed a functional repeat polymorphism. We used a larger patient group, to reach enough statistical power. This larger group included the patients of which intragraft mRNA was measured. However, we did not find any association between the length of the repeat, thus genetic production capacity, and the development of TCAD. Processes around the transplantation procedure probably overrule the potential effect of the polymorphism. Alternatively, the reported *in vitro* associations between the length of the repeat and the promoter activity do not apply for the *in vivo* situation after heart transplantation. Moreover, the immunosuppressive therapy used might abrogate the effect of the polymorphism on the production of HO-1. Furthermore, the use of HMG-CoA-reductase inhibitors was higher in the TCAD negative group. These inhibitors have

anti-inflammatory and immunosuppressive properties and might have influenced the development of TCAD, thereby overruling the effect of the polymorphism.[23] Also, the total cholesterol levels were higher in the TCAD positive group, which might have more influence on the development of TCAD than the HO-1 polymorphism.

We are aware of the limitations of our study. Our patient groups in which we measured mRNA expression are small and the biopsies taken at the different time points are not from a consistent cohort of patients. Nevertheless, consistent data for HO-1, TGF-ß and Bcl-2/Bax balance were generated. Furthermore, visual angiographic assessment of the coronary arteries instead of intravascular ultrasound (IVUS) was used to define the presence of abnormalities in the coronary arteries. Visual assessment of angiograms is less sensitive as not the vessel wall but the contrast filled lumen is assessed. Measurements of vessel wall abnormalities by IVUS would have been preferable. Unfortunately, such procedure is not routinely performed at our institution. A possible misdiagnosis might be an explanation for the dichotomy in the mRNA expression levels of HO-1 at week one in the TCAD positive patients.

In conclusion, in our study, HO-1 expression is associated with TCAD. Although it is not clear whether the increase in HO-1 mRNA expression is the cause or the consequence of TCAD, our data suggest that upregulation of HO-1 is an adaptive response to limit cell and tissue injury and a subsequent inflammation process. The overexpression of HO-1 might therefore be a reflection of damage due to the transplantation procedure and the subsequent inflammatory response. To obtain the same protective results as in experimental transplant setting, induction of HO-1 already in the donor might be necessary. However, it is well possible that induction of only one protective gene is not enough to prevent development of a complex disease as TCAD after clinical heart transplantation.

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Chapter 9

The functional Heme Oxygenase-1 promoter polymorphism in relation to heart failure and complications after cardiac transplantation

Cécile Holweg
Aggie Balk
André Uiterlinden
Bert Niesters
Lex Maat
Willem Weimar
Carla Baan

Submitted

Abstract

Heme oxygenase (HO)-1 is expressed in response to oxidative stress, which is associated with the development of native (CAD) and transplant coronary artery disease (TCAD) and acute rejection (AR). The enzymatic activity of the protective enzyme HO-1 is inversely related to the number of repeats of the $(GT)_n$ polymorphism in the promoter. Thus, this polymorphism might explain individual differences in susceptibility to oxidative stress induced injury. Therefore, the $(GT)_n$ repeat in the HO-1 gene was studied in relation to CAD, TCAD and AR. Alleles were divided in 3 groups, short (high producers), medium (intermediate producers) and long (low producers), and in two groups (high and low producers). We compared patients with ischemic heart disease (n=167) and dilated cardiomyopathy (n=129). Allele or genotype frequencies were not significantly different between both groups. After transplantation, recipients without and with AR (recipients: n=63 vs n=241; donors: n=50 vs n=203), and without and with TCAD (recipients: n=207 vs n=85; donors: n=169 vs n=74) were compared. No associations with the repeat were found for either recipients or donors in relation to AR or TCAD. We conclude that the functional HO-1 $(GT)_n$ polymorphism is not associated with the primary disease (CAD) nor with development of AR or TCAD after heart transplantation.

Introduction

Oxidant production is part of our defense strategies aiming at the destruction of pathogens. However, these reactive oxygen species (ROS) can have damaging effects on healthy tissue.[1] ROS induces apoptosis and necrosis of endothelial cells, vascular smooth muscle cells and cardiac myocytes. It also enhances pro-inflammatory cytokine production by activation of Nuclear Factor κB (NFκB). Therefore, the presence of oxidants might lead to activation of the immune system, enhancement of pro-inflammatory cytokine production and subsequently to inflammatory responses.[1, 2]

Endothelial injury induced by oxidative stress might be involved in the pathogenesis of coronary artery disease (CAD) and in myocardial ischemia.[3, 4] Damage of endothelial cells leads to dysfunction of these cells, which can elicit cellular interactions that finally result in the formation of atherosclerotic lesions.[5] Oxidative stress induced injury might also be an important trigger for the development of complications after heart transplantation, like acute rejection (AR) and transplant coronary artery disease (TCAD).[6, 7] Cell damage due to peri-transplant processes leads to upregulation of HLA-antigen and adhesion molecules on endothelial cells, which then promotes migration of host leukocytes into the graft. This subsequently might lead to allogen-dependent processes like AR and to inflammatory responses finally resulting in TCAD.[8]

The deleterious effects of oxidant stress are usually prevented by anti-oxidant defense mechanisms, of which the Heme Oxygenase (HO)-1 is one.[9] The cytoprotective enzyme HO-1 is found to be expressed by many cells and tissues in response to oxidative injury. HO-1 catalyzes the degradation of heme into biliverdin, iron and carbon monoxide (CO). Biliverdin is subsequently converted to bilirubin and iron into ferritin. The end products have anti-oxidant functions and are thought to be responsible for the protective effects attributed to HO-1, such as anti-inflammatory, anti-apoptotic and anti-proliferative effects.[3] Biliverdin and bilirubin both scavenge oxygen radicals and prevent the oxidation of LDL. CO is able to inhibit pro-inflammatory cytokine and chemokine production and leukocyte infiltration. CO also modulates cell cycle progression, thereby inhibiting cell proliferation and regulates apoptosis.[10, 11]

The enzymatic activity of HO-1 *in vitro* is genetically controlled by a $(GT)_n$ repeat polymorphism in the promoter, whereby the number of repeats is inversely related to the activity.[12, 13] It can be speculated that individuals with a genetic capacity for lower HO-1 activity, are more prone for oxidative stress induced injury and, thus, for atherosclerotic CAD and complications after heart transplantation, like

AR and TCAD. Indeed, associations between the HO-1 repeat and coronary artery disease (CAD) in a Chinese patient population with type 2 diabetes and in a Japanese patient population with other risk factors for CAD, like hypercholesterolemia, diabetes and smoking have been reported. In both studies, patients carrying longer repeats were more susceptible for development of CAD.[13,14]

To our knowledge, studies between the HO-1 repeat polymorphism in relation to heart transplantation have not been published. In the present study, we attempted to identify patients predisposed for native or transplant CAD or development of AR, in relation to this particular polymorphism.

Material and methods

Patients

We genotyped DNA of 311 out of 329 consecutive heart transplant recipients, who were transplanted in Rotterdam between June 1984 and January 1998. In addition, DNA of 263 cardiac donors was genotyped. All patients gave permission to use their material and data for research purposes. Data of 310 patients were available for analysis of the HO-1 repeat in relation to the primary disease. Of these 310 patients, 167 were transplanted because of ischemic heart disease, 129 because of dilated cardiomyopathy and 14 for other reasons. Because of the small number, the last group has been excluded from the analyses. All patients were in NYHA class III or IV at time of transplantation and without contra-indications for heart transplantation, i.e., active infection, irreversible pulmonary hypertension, severe kidney or liver dysfunction, diabetes mellitus with secondary organ damage, other systemic disease or severe peripheral vascular disease.

Table 1. Characteristics of patients before transplantation

Primary disease	IHD	CMP	p-value
No of patients (n)	167	129	
Age (years)*	51.1±7.6	43.2±13.6	<0.001[a]
Gender (M/F)	156/11	90/39	<0.001[b]

CMP: cardiomyopathy; IHD ischemic heart disease. *Years are expressed as mean ± SD. P-values were obtained by Mann-Whitney[a] and χ^2-test[b].

For analysis of the HO-1 repeat in relation to acute rejection in the first post transplant year, data of 304 patients and 253 donors were available. AR was histologically diagnosed in endomyocardial biopsies according to the criteria of the International Society for Heart and Lung Transplantation (ISHLT ≥3A).[15] Of the 304 recipients, 63 had no AR (DNA of 50 donors was available), 241 experienced one or more AR (DNA of 203 donors was available).

Table 2. Characteristics of patients with and without acute rejection.

	No AR	AR	p-value
No. of patients	63	241	
Recipient age (years)*	44.9±13.1	48.0±11.0	0.15†
Donor age (years)*	25.5±8.8	27.1±9.5	0.25†
Recipient gender (M/F)	55/8	200/41	0.56‡
Donor gender (M/F)	50/13	132/109	0.0005‡
Original heart disease			
CMP/IHD/other	30/32/1	96/131/13	0.30‡
Cold ischemia time (min)*	174±41	165±80	0.10**
HLA-AB mismatches*	3.0±0.9	3.0±0.9	0.93†
HLA-DR mismatches*	1.3±0.7	1.5±0.6	0.03†
Induction therapy			
None/ATG/OKT3/anti-CD25	19/17/21/6	62/65/74/40	0.55‡
CMV infection (no/yes)	35/28	134/107	0.99‡
CMV disease (no/yes)	55/8	198/43	0.45‡
HMG-CoA-reductase inhibitors (no/yes)	61/2	215/26	0.08‡

AR: acute rejection, IHD: ischemic heart disease, CMP: dilated cardiomyopathy, CMV: cytomegalovirus. *Mean ± SD. P-values were obtained by **Student's t-test, †Mann-Whitney-test and ‡(exact) χ^2-test.

When we analyzed the HO-1 repeat polymorphism in relation to TCAD, data of 292 patients and 243 donors were available. TCAD was diagnosed at one year after transplantation by visual assessment of the coronary angiogram and defined as all abnormalities of the epicardial as well as the intramyocardial arteries, including minimal irregularities. [16] Of the 292 patients 85 were diagnosed positive (DNA of 74 of their donors was available) and 207 negative (DNA of 169 donors).

The patient and donor population consisted mainly (>95%) of Caucasians. All patients received cyclosporin A and low-dose of steroids as maintenance immunosuppressive therapy. Cyclosporin A was aimed to keep trough levels between 250-350 ng/ml in the first 6 months after transplantation and between 100-200 ng/ml after 6 months.

Patient characteristics for primary disease i.e. recipient age and gender are summarized in table 1. Patient demographics for AR, i.e. recipient and donor age, recipient and donor gender, primary disease, cold ischemia time, induction

therapy, and the number of HLA mismatches are summarized in table 2 and for TCAD the same variables as for AR and in addition the number of AR in the first year, in table 3.

Table 3. Characteristics of patients with and without TCAD

	TCAD neg	TCAD pos	p-value
No.	207	85	
Recipient age (years)*	46.5±11.7	49.4±10.7	0.05†
Donor age (years)*	25.3±8.8	30.±9.6	<0.0001**
Recipient gender (M/F)	175/32	71/14	0.86‡
Donor gender (M/F)	126/81	52/33	1.0‡
Original heart disease			
CMP/IHD/other	92/106/9	28/51/5	0.21‡
Cold ischemia time (min)*	165±38	171±41	0.27**
HLA-AB mismatches*	3.0±0.9	2.9±0.9	0.65†
HLA-DR mismatches*	1.4±0.6	1.5±0.6	0.23†
Induction therapy			
None/ATG/OKT3/anti-CD25	57/53/70/27	19/26/22/18	0.17‡
No of AR in the first year*	1.7±1.5	2.0±1.7	0.25†
CMV infection (no/yes)	114/93	47/38	1.0‡
CMV disease (no/yes)	172/35	71/14	1.0‡
Total cholesterol (nmol/L)*	7.1±1.7	7.6±2.3	0.04**
Triglycerides (nmol/L)*	2.4±1.1	2.5±1.3	0.71†
HMG-CoA-reductase inhibitors (no/yes)	190/70	75/10	0.0046‡

TCAD: transplant coronary artery disease, CMP: dilated cardiomyopathy, IHD: ischemic heart disease, AR: acute rejection. *Mean ± SD. p-values were obtained by **Student's t-test, †Mann-Whitney-test and ‡(exact) χ^2-test.

DNA isolation for genotyping

DNA was isolated from 10^6 patient peripheral blood mononuclear cells (PBMC), by the method described by Boom *et al.*[17] The vast majority of PBMC were collected before transplantation. Cells were lysed in a buffer containing Triton X-100 and guanidinium-iso-thiocyanate. Thereafter, the DNA was bound to celite, washed twice with a buffer containing guanidinium-iso-thiocyanate, twice with 70% ethanol and once with acetone. The pellet was vacuum dried and dissolved in 100 µl double distillated water. DNA was eluted by incubation at 56°C for 10 min. After centrifugation, the supernatant, containing the genomic DNA, was removed and placed into a clean tube.

Genotyping of the $(GT)_n$ repeat polymorphism in the HO-1 promoter

Genotyping of the repeat in the promoter region of the HO-1 gene was performed by PCR followed by fragment analysis of the amplified PCR product. For PCR, we used a 5' FAM labeled forward primer (5'-FAM-AGA GCC TGC AGC TTC TCA GA-3') and a reverse primer (5'-ACA AAG TCT GGC CAT AGG AC-3') covering the GT repeat. The PCR mixture contained 0.2 mM of each dATP, dTTP, dCTP, dGTP, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.5 µM of each primer and 1.5 U AmpliTaq GOLD polymerase (Applied Biosystems). PCR was performed in a GeneAmp® PCR System 9700 (Applied Biosystems) and samples were subjected to 40 cycles of denaturation at 94°C for 20 sec, annealing at 60°C for 10 sec and extension at 72°C for 20 sec. The last cycle was extended with 7 min at 72°C. Fragment analysis was performed using an ABI Prism™ 310 Genetic analyzer with Genescan™ Analysis software 1.2 (Applied Biosystems). TAMRA 350 (Applied Biosystems) was used as internal size standard.

The allele sizes observed were confirmed by sequencing DNA of homozygous patients.

Statistics

Evaluation of statistical differences in characteristics between patients with and without acute rejection was performed by Student's T-test and Mann Whitney test for continues variables and (exact) χ^2-test for discrete variables, as appropriate.

To study possible associations between the repeat polymorphism and primary disease, AR and TCAD, alleles were grouped into 3 classes, according to Yamada et al., short alleles (S: <27 repeats), medium alleles (M: 27–32 repeats) and long alleles (L: >32 repeats). (12) In addition, we analyzed the alleles divided in two groups, small alleles (S: <27 repeats) and long alleles (L: ≥27 alleles) as performed by Kaneda et al.(14) All of these comparisons were done using the (exact) χ^2-test. P-values ≤ 0.05 were considered to be significant.

Results

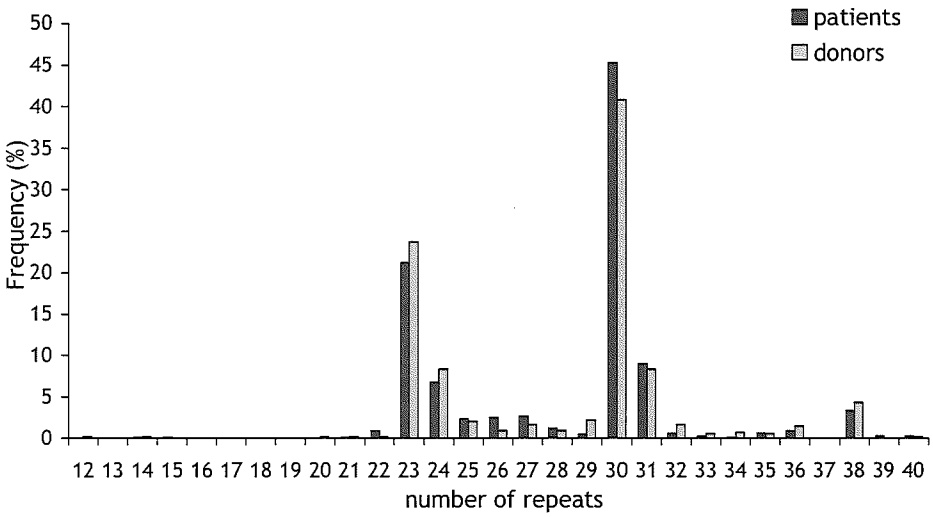
Patient characteristics

Patient characteristics between patients with IHD and CMP were different. The mean age of patients was significantly higher in the patients with IHD than in CMP patients. This group also contained less female patients compared to the CMP group.

For the patient characteristics of patients with or without AR, we found a significant higher number of female donors and a higher number of HLA-DR mismatches in the patient group with AR. The other variables were comparable.
The patient characteristics of patients with and without TCAD were comparable between the groups, except for recipient and donor age, total cholesterol level and the use of statins. Both the patient and donor age were higher in the TCAD positive group compared to the negative group. Also the mean total cholesterol level was higher in this group. The use of HMG-CoA reductase inhibitors (statins) was higher in the TCAD negative group.

Analysis of the HO-1 repeat polymorphism

We observed 23 alleles ranging from 12 repeats to 40 repeats (Figure 1) To analyze the alleles in relation to reason for transplantation (primary disease), AR en TCAD, we grouped the alleles as described in the materials and methods section.



Allele distribution of the $(GT)_n$ repeat polymorphism in the promoter of the HO-1 gene in heart transplant recipients and their donors.

We first analyzed the repeat polymorphism in relation to the primary disease of the cardiac recipients, according to three allele categories, S, M, L. No associations were found for either the allele frequencies or genotype distribution when this classification was used (Table 4). Subsequently, we analyzed the repeat according to the two allele size categories described in the statistics section of materials and methods, but this again did not result in an association. The results are shown in Table 4.

Table 4. Heme Oxygenase-1 $(GT)_n$ repeat in relation to the primary disease of the heart transplant patients

	Primary disease		
	IHD n (%)	CMP n (%)	p-value
Alleles (3 groups)			
S (<27)	119 (35.6)	79 (30.6)	
M (27-32)	194 (58.1)	163 (63.2)	0.42
L (>32)	21(6.3)	16 (6.2)	
Genotypes			
S/S	22 (13.2)	16 (12.4)	
S/M	67 (40.1)	43 (33.3)	
S/L	8 (4.8)	4 (3.1)	0.57*
M/M	59 (35.5)	54 (41.9)	
M/L	9 (5.4)	12 (9.3)	
L/L	2 (1.2)	0 (0)	
Alleles (2 groups)			
S (<27)	119 (35.6)	79 (30.6)	0.22
L (≥27)	215 (64.4)	179 (69.4)	
Genotypes			
S/S	22 (13.2)	16 (12.4)	
S/L	75 (44.9)	47 (36.4)	0.26
L/L	70 (41.9)	66 (51.2)	

IHD: ischemic heart disease, CMP: dilated cardiomyopathy, S: short alleles, M: medium alleles, L: long alleles. Comparisons were done using the (exact) χ^2-test. P-values ≤ 0.05 were considered to be significant. *For statistical analysis, the L/L genotype was combined with the M/L genotype.

After transplantation, we studied the repeat in relation to AR after heart transplantation and analyzed the allele and genotype frequencies of both recipients and donors. Again we first used the allele categories defined by Yamada *et al.* and then by Kaneda *et al.*[12, 14] Neither for the recipient nor for the donor, we observed statistical differences between the distribution of the alleles or genotypes for any of the analyzed allele categories and AR (Table 5).

Finally, we analyzed the allele and genotype distribution in relation to TCAD. Also here, we analyzed both recipient and donor DNA. Again, we did not find associations for any of the allele categories for either the recipient or donor. The allele and genotype frequencies are given in Table 6.

Table 5. Heme Oxygenase-1 $(GT)_n$ repeat in relation to acute rejection after heart transplantation

	Recipient			Donor		
	no AR n (%)	AR n (%)	p-value	no AR n (%)	AR n (%)	p-value
Allele (3 groups)						
S (<27)	43 (34.1)	166 (34.4)		40 (40.0)	142 (35.0)	
M (27-32)	71 (56.3)	291 (60.4)	0.19	55 (55.0)	232 (57.1)	0.46
L (>32)	12 (9.5)	25 (5.2)		5 (5.0)	32 (7.9)	
Genotype						
S/S	6 (9.5)	36 (14.9)		6 (12.0)	22 (10.8)	
S/M	26 (41.3)	87 (36.1)		25 (50.0)	89 (43.8)	
S/L	5 (7.9)	7 (2.9)	0.22*	3 (6.0)	9 (4.4)	0.68*
M/M	20 (31.7)	94 (39.0)		14 (28.0)	63 (31.0)	
M/L	5 (7.9)	16 (6.6)		2 (4.0)	17 (8.4)	
L/L	1 (1.6)	1 (0.4)		0 (0)	3 (1.5)	
Alleles (2 groups)						
S (<27)	43 (34.1)	166 (34.4)	1.0	40 (40.0)	142 (35.0)	0.35
L (≥27)	83 (56.9)	316 (65.6)		60 (60.0)	264 (65.0)	
Genotypes						
S/S	6 (9.5)	36 (14.9)		6 (12.0)	22 (10.8)	
S/L	31 (49.2)	94 (39.0)	0.28	28 (56.0)	98 (48.3)	0.51
L/L	26 (41.3)	111 (46.1)		16 (32.0)	83 (40.9)	

AR: acute rejection, S: short alleles, M: medium alleles, L: long alleles. Comparisons were performed using the (exact) χ^2-test. P-values ≤ 0.05 were considered to be significant. *For statistical analysis, the L/L genotype was combined with the M/L genotype.

Discussion

We tried to identify patients predisposed for atherosclerotic heart disease in the native heart, coronary artery disease in the transplanted heart and acute rejection after transplantation, using the variable number of tandem repeat repeat polymorphism $(GT)_n$ in the gene of the protective enzyme HO-1. This HO-1 promoter polymorphism $(GT)_n$ has been associated with the enzymatic activity of HO-1 and a relation was found with various cardiovascular disorders and also with susceptibility for emphysema and neurodegenerative diseases.[12-14, 18-20] A common feature of these disorders is that oxidative stress is involved in their pathogenesis. Oxidant induced injury is also thought to be implicated in CAD and transplant related complications. However, we did not observe associations

Table 6. Heme Oxygenase-1 $(GT)_n$ repeat in relation to transplant coronary artery disease

	Recipient			Donor		
	TCAD neg n (%)	TCAD pos n (%)	p-value	TCAD neg n (%)	TCAD pos n (%)	p-value
Allele (3 groups)						
S (<27)	147 (35.5)	53 (31.2)	0.49	122 (36.1)	52 (35.1)	0.74
M (27-32)	241 (58.2)	108 (63.5)		193 (57.1)	83 (56.1)	
L (>32)	26 (6.3)	9 (5.3)		23 (6.8)	13 (8.8)	
Genotype						
S/S	31 (15.0)	10 (11.8)		20 (11.8)	8 (10.8)	
S/M	77 (37.2)	30 (35.5)		74 (43.8)	33 (44.6)	
S/L	8 (3.9)	3 (3.5)	0.75*	8 (4.7)	3 (4.1)	1.0*
M/M	74 (35.7)	37 (43.5)		52 (30.8)	23 (31.1)	
M/L	16 (7.7)	4 (4.7)		15 (8.9)	4 (5.4)	
L/L	1 (0.5)	1 (1.2)		0 (0)	3 (4.1)	
Alleles (2 groups)						
S (<27)	147 (35.5)	53 (31.2)	0.34	122 (36.1)	52 (35.8)	0.92
L (≥27)	267 (64.5)	117 (68.8)		216 (63.9)	96 (64.9)	
Genotypes						
S/S	31 (15.0)	10 (11.8)	0.63	20 (11.8)	8 (10.8)	0.97
S/L	85 (41.1)	33 (38.8)		82 (48.5)	36 (48.6)	
L/L	91 (44.0)	42 (49.4)		67 (39.6)	30 (40.5)	

TCAD: transplant coronary artery disease, S: short alleles, M: medium alleles, L: long alleles. Comparisons were done using the (exact) χ^2-test. P-values ≤ 0.05 were considered to be significant. *For statistical analysis, the L/L genotype was combined with the M/L genotype.

between the HO-1 repeat and the primary diseases of patients who received a cardiac transplant, the occurrence of AR after transplantation or the development of TCAD. HO-1 is only one protective gene and other protective mechanisms might be involved in the development of native and transplant CAD and AR after cardiac transplantation. For example, enzymes like superoxide dismutase, catalase, gluthathion peroxidase and the inducible form of nitric oxide synthase or protective genes, such as the Bcl-2, Bcl-x_L, A1 and A20 can be candidates.[2, 21, 22] We speculate that a genetic capacity for lower HO-1 activity might be compensated by other protective genes or enzymes. In addition, CAD and transplant related complications are multifactorial disorders and can not be defined by only one gene or one gene polymorphism. Several protective mechanisms might act simultaneously and thereby overrule the effect of the HO-1 polymorphism.

The fact that we were not able to find an association between the HO-1 repeat polymorphism and CAD, but other groups were, can also be due to the fact that

the chosen end-points for CAD were not similar. Indeed, both associations were found in a subgroup of patients with CAD, i.e., in patients with type 2 diabetes and in patients who also have other coronary risk factors, such as diabetes, hypercholesterolemia and smoking. In both studies, no associations were found when the total patient groups with and without CAD were compared.[13, 14]

Also the differences in patient characteristics between the groups might have influenced the outcomes of the analyses with the repeat polymorphism. For example, HLA mismatches influence the development of AR and the higher number of HLA-DR mismatches in the patient group with AR compared to the group without AR is probably of more importance than the HO-1 repeat polymorphism. In the studied patients with and without TCAD, the total cholesterol levels were higher in the TCAD positive group, which might diminish the effect of the polymorphism on the development of TCAD. Another explanation for the lack of association might be that the reported *in vitro* associations between the length of the repeat and the promoter activity might not apply for the *in vivo* situation. Moreover, the used medication for heart failure and immunosuppressive drugs after transplantation might abrogate the effect of the polymorphism on the production of HO-1. For example, steroids inhibit activation of NFκB, which is not only a transcription factor for pro-inflammatory cytokines, but also for HO-1 and, thus, might influence HO-1 expression.[23, 24] In this perspective, the genetically determined sensitivity of patients for immunosuppressive agents plays a more important role than the polymorphism in the HO-1 gene. In addition, the use of statins was higher in the TCAD negative group. Statins have anti-inflammatory and immunosuppressive properties and might thus have influenced the development of TCAD and thereby have overruled the effect of the polymorphism.[25]

We conclude that the functional $(GT)_n$ repeat polymorphism in the promoter of the HO-1 gene is not associated with ischemic heart disease as the cause of heart failure requiring transplantation nor to the development of AR or TCAD after heart transplantation in our patient population.

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Chapter 10

Gene polymorphisms in heart transplantation: an overview and discussion of the current literature

Cécile Holweg
Willem Weimar
André Uitterlinden
Carla Baan

Based on a part of review:
Clinical impact of cytokine gene polymorphisms in heart and lung transplantation
Journal of Heart and Lung Transplantation, in press

Introduction

Inflammatory responses play a role in the pathogenesis of heart failure leading to transplantation and also in transplant related complications. A number of factors implicated in immune and inflammatory reactions are genetically controlled by polymorphisms. Gene polymorphisms might thus influence the development of various diseases leading to heart failure and could be responsible for differences in susceptibility to complications after transplantation, time to onset or the severity of a complication, but also in the way a recipient responds to drug therapy or immunosuppressive therapy or to treatment of transplant related problems. Studying frequencies and patterns of polymorphisms, in genes known to be involved in heart failure or transplant related complications, in recipients, organ donors and controls, can give us information about processes in the development and progression of these disorders and to identify potential genetic risk factors. In transplantation medicine specific attention has been paid to variations in the genes encoding cytokines and recently by our group also to a polymorphism in the stress protein heme oxygenase (HO)-1.
In this review, we aim to give an overview of the literature, including our own results, and discuss the results of these studies. We tried to ascertain whether these studies together have already resulted in the identification of genetic risk factors for the cause of heart failure leading to transplantation or for transplant related complications and whether we were able to use these potential risk factors in the clinical practice. In other words, can we already give practical guidelines for the monitoring and treatment of our patients, with the results of genetic association studies obtained so far.

Gene polymorphisms in end-stage heart failure

Cardiovascular disease is an important cause of death in the Western world that can lead to end-stage heart failure, for which heart transplantation is the only treatment. Most patients who are referred for heart transplantation suffer from heart failure due to ischemic heart disease (IHD) or dilated cardiomyopathy (DCM) and some to valvular heart disease. As heart failure is thought to be an inflammatory disease, the balance between pro- and anti- inflammatory cytokines can be important. Indeed, this balance is disturbed in patients with severe heart failure. For example, circulating TNF-α levels are elevated in patients with end-stage heart failure. IL-10 can inhibit TNF-α production, but IL-10 levels apparently

were not adequately raised in these patients. Also a role is attributed to cytokines like IL-2, IFN-γ and IL-4. This role is probably in regulating the inflammatory response. IL-2 and IFN-γ increase the inflammatory response, while Il-4 is thought to suppress this reaction.[1-3] In relation to heart failure leading eventually to cardiac transplantation, only 7 studies describing polymorphisms in 5 genes are published by only 3 groups. We summarize these results in table 1 and the positive associations are outlined below.

In the TGF-ß gene, only the SNP at position +869 T/C was reported to be associated with the original disease of patients who received a heart transplant. An excess of heterozygous was observed in individuals with DCM compared to patients with IHD or controls.[4] However, this finding could not be confirmed by two other groups.[5, 6]

Densem and colleagues studied a polymorphism at position -308 (A/G) in the promoter of the TNF-α gene in relation to heart failure. They found an overrepresentation of the A allele in the subgroup of patients with a pretransplant diagnosis of viral mediated or idiopathic myocardial dysfunction in comparison of patients with IHD and controls.[7]

Table 1. Cytokine and Heme Oxygenase-1 gene polymorphisms in relation to primary diseases of heart transplant recipients

Gene	Polymorphism	Location	Number of patients and controls	Association	Ref
TGF-ß1	+869 T/C (Leu^{10}→Pro)	Leading sequence	253 patients, 94 controls	Yes	4
TGF-ß1	+869 T/C (Leu^{10}→Pro)	Leading sequence	175 patients, 268 controls	No	6
TGF-ß1	+869 T/C (Leu^{10}→Pro)	Leading sequence	64 patients, 629 controls from ref 38	No	5
TGF-ß1	+915 G/C (Arg^{25}→Pro)	Leading sequence	253 patients, 94 controls	No	4
TGF-ß1	+915 G/C (Arg^{25}→Pro)	Leading sequence	175 patients, 268 controls	No	6
TGF-ß1	+915 G/C (Arg^{25}→Pro)	Leading sequence	64 patients, 629 controls from ref 38	No	5
TGF-ß1	-509 C/T	promoter	64 patients, 629 controls from ref 38	No	5
TGF-ß1	-800 G/A	promoter	64 patients, 629 controls from ref 38	No	5
TNF-α	-308 A/G	promoter	175 patients, 212 controls	Yes	7
IL-10	-592 C/A	Promoter	64 patients, 629 controls from ref 38	No	39
IL-10	-819 C/T	Promoter	64 patients, 629 controls from ref 38	No	39
IL-10	-1082 C/T	Promoter	64 patients, 629 controls from ref 38	No	39
IL-4	-81 A/G	Promoter	64 patients, 629 controls from ref 38	No	17
IL-4	-285 C/T	Promoter	64 patients, 629 controls from ref 38	No	17
IL-4	-590 C/T	Promoter	64 patients, 629 controls from ref 38	No	17
HO-1	GT repeat	Promoter	269 patients	No	44

For associations, see text for details.

Gene polymorphisms in heart transplantation

Acute rejection

Acute rejection is an inflammatory process that is characterized by infiltration of mononuclear cells leading to myocyte damage or even necrosis. Acute rejection after heart transplantation is diagnosed by histological examination of routinely taken endomyocardial biopsies.[8] Cytokines, such as TNF-α, IL-2, IFN-γ and IL-4, are involved in the pathogenesis of acute rejection.[9-11] In relation to acute cardiac rejection, many cytokine and cytokine receptor gene polymorphisms of both recipient and donor origin are studied. Table 2 shows the results for 9 genes in which 27 different polymorphisms were studied of which some by several groups. Of these polymorphisms, 7 resulted in a genetic association that we here describe in detail.

For the TNF-α gene, a SNP in the promoter at position -308 A/G is found to be associated with rejection in three patient groups. This SNP is functional, and it is supposed that carriers of an A-allele produce more TNF-α than individuals without an A-allele.[12] The A-allele of this SNP was found to be a risk factor for acute rejection.[13-15] Turner and colleagues found this TNF-α SNP to be related to acute rejection when present in combination with an A allele of a SNP at position -1082 in the IL-10 gene.[15] This association between acute rejection and the combination of an A-allele of TNF-α -308 SNP and an A-allele of the IL-10 -1082 SNP was also found in a pediatric population, described by Awad et al.[14] This group also analyzed the combination of three SNP's in the IL-10 promoter (-1082 G/A, -819 C/T and -592 C/A) and found that patients homozygous for the A-allele at position -1082 were more often rejectors than G-allele carriers.[14] This -1082 A-allele in the IL-10 SNP has been associated with lower IL-10 production compared to a G-allele.[16] The results of these two studies indicate that carrying the TNF-α -308 A-allele combined with the IL-10 -1082 A-allele might be a potential risk factor for development of acute rejection.

Of a repeat polymorphism $(CA)_m(CT)_n$ in the 3'flanking region of IL-2 gene, it is not known whether it is functional or in linkage with a functional SNP. For this repeat, we found a significant association between a recipient allele (135) of the repeat polymorphism $(CA)_m(CT)_n$ in the 3'flanking region of the gene and freedom from acute rejection. Moreover, an interaction was found between this allele, the number of HLA-DR mismatches and acute rejection. The frequency of patients with AR was significantly lower in patients carrying the 135 allele and zero or one HLA-DR mismatch compared to patients with two mismatches or patients without the allele.

Table 2. Cytokine and Heme Oxygenase-1 gene polymorphisms in heart transplant recipients and their donors in relation to acute rejection

Gene	Polymorphism	Location	Number of recipients, donors or controls	Association	Ref
TNF-α	-308 A/G	Promoter	119 recipients	Yes	13
TNF-α	-308 A/G	Promoter	115 recipients	Yes	15
TNF-α	-308 A/G	Promoter	93 recipients, 29 donors	Yes	14
TNF-α	-308 A/G	Promoter	62 recipients	No	19
TNF-α	-376 G/A	Promoter	62 recipients	No	19
TNF-α	-238 G/A	Promoter	62 recipients	No	19
TNF-α	TC repeat (TNFd)	3' flanking region	62 recipients	No	19
TNF-α	TC repeat (TNFe)	3' flanking region	62 recipients	No	19
TGF-ß1	+869 T/C (Leu^{10}→Pro)	Leading sequence	93 recipients, 29 donors	No	14
TGF-ß1	+869 T/C (Leu^{10}→Pro)	Leading sequence	70 recipients, 61 donors	No	5
TGF-ß1	+915 G/C (Arg25Pro)	Leading sequence	93 recipients, 29 donors	No	14
TGF-ß1	+915 G/C (Arg^{25}→Pro)	Leading sequence	70 recipients, 61 donors	No	5
TGF-ß1	-509 C/T	Promoter	70 recipients, 61 donors	No	5
TGF-ß1	-800 G/A	Promoter	70 recipients, 61 donors	No	5
IL-10	-1082 G/A	Promoter	115 recipients	Yes	15
IL-10	-1082 G/A	Promoter	93 recipients, 29 donors	Yes	14
IL-10	-1082 G/A	Promoter	70 recipients, 60 donors	No	39
IL-10	-819 C/T	Promoter	115 recipients	No	15
IL-10	-819 C/T	Promoter	93 recipients, 29 donors	Yes	14
IL-10	-819 C/T	Promoter	70 recipients, 60 donors	No	39
IL-10	-592 C/A	Promoter	115 recipients	No	15
IL-10	-592 C/A	Promoter	93 recipients, 29 donors	Yes	14
IL-10	-592 C/A	Promoter	70 recipients, 60 donors	No	39
IFN-γ	CA repeat	First intron	301 recipients	No	40
IFN-γ	+874 A/T	First intron	93 recipients, 29 donors	No	14
IL-2	-330 T/G	Promoter	301 recipients	No	40
IL-2	$(CA)_m(CT)_n$ repeat	3' flanking region	290 recipients, 101 controls	Yes	41
IL-6	-174 G/C	Promoter	93 recipients, 29 donors	No	14
IL-4	-285 C/T	Promoter	70 recipients, 61 donors, 36 controls	No	17
IL-4	-590 C/T	Promoter	70 recipients, 61 donors, 36 controls	Yes	17
IL-4	-81 A/G	Promoter	70 recipients, 61 donors, 36 controls	No	17
ICAM-1	E469/K469 (C/T)	Exon 6	82 recipients, 96 donors, 101 controls	Yes	18
ICAM-1	G241/R241 (C/T)	Exon 4	82 recipients, 96 donors, 101 controls	No	18
TNF-ß	AC repeat (TNFa)	5' flanking region	62 recipients	Yes	19
TNF-ß	AspHI	First intron	62 recipients	No	19
TNF-ß	Nco1	First intron	62 recipients	No	19
TNF-ß	TC repeat (TNFb)	5' flanking region	62 recipients	No	19
TNF-ß	TC repeat (TNFc)	First intron	62 recipients	No	19
HO-1	GT repeat	Promoter	304 recipients, 253 donors	No	44

For associations, see text for details.

An IL-4 promoter SNP (-590 C/T) was related with a lower frequency of acute rejection. Carriers of a donor T-allele and patients with a combination of a donor T-allele and without a patient T-allele experienced less acute rejection episodes.[17] This T-allele increases the *in-vitro* IL-4 promoter activity relative to the C-allele.
In the adhesion molecule ICAM-1, a SNP (C/T) located in exon 6, changing the amino acid at codon 469 from Glutamic acid (E) to Lysine (K, E469K) was also associated with acute rejection. For both SNP's, the possible functionality is currently unknown. A higher frequency of the recipient Lys469 allele was found in patients with persistent acute rejection than in patients without persistent rejection or in controls, suggesting this to be a risk allele.[18]
Finally, in the TNF-ß gene, a CA repeat in the 3'flanking region was found to be associated with acute rejection, whereby carriers of allele 10 (out of 13 alleles observed) had more frequent rejection episodes than non-carriers.[19] Again, the functionality of this polymorphism or linkage with a functional polymorphism is not clear.

Transplant coronary artery disease

Transplant coronary artery disease (TCAD) is a chronic inflammatory process characterized by progressive and diffuse concentric intimal thickening of the entire length of the donor vessels, including the epicardial as well as the intramyocardial arteries. This intimal thickening is due to migration of macrophages, migration and proliferation of T-cells and smooth muscle cells, collagen accumulation and fibrosis.[20] The initiation and progression of TCAD is partially immune mediated. Polymorphisms, of both recipient and donor, in cytokine genes were studied in an attempt to associate them to TCAD. Twenty-two different polymorphisms in 12 genes were studied, seven polymorphisms of which were observed to be associated with this complication. These associations are described while the remainders are summarized in Table 3.
One of the associated polymorphisms with TCAD is the SNP in the TGF-ß1 gene at position +915 G/C, changing the amino acid at codon 25 from Arg to Pro. This Arg allele is believed to be responsible for higher TGF-ß1 production capacity compared to the Pro allele.[21] In two study populations homozygosity for Arg at codon 25 was found to be a significant risk factor for the development of TCAD [22, 23], but this could not be confirmed in two other studies.[24, 25] However, in one of them it was found that the SNP at +869 T/C, which alters amino acid 10 from Leu to Pro, the Pro allele at codon 10 was related to TCAD. Patients homozygous for the Pro allele had the highest risk to develop TCAD.[24] This Leu^{10}→Pro SNP is also thought to influence the TGF-ß1 production, although it is to date not clear

Table 3. Cytokine and Heme Oxygenase-1 gene polymorphisms in heart transplant recipients and their donors in relation to transplant coronary artery disease

Gene	Polymorphism	Location	Number of recipients, donors, or controls	Association	Ref
TGF-ß1	+869 T/C (Leu^{10}→Pro)	Leading sequence	236 recipients, 197 donors	Yes	24
TGF-ß1	+915 G/C (Arg^{25}→Pro)	Leading sequence	236 recipients, 197 donors	No	24
TGF-ß1	+915 G/C (Arg^{25}→Pro)	Leading sequence	129 recipients	Yes	23
TGF-ß1	+915 G/C (Arg^{25}→Pro)	Leading sequence	175 recipients	Yes	22
TGF-ß1	+915 G/C (Arg^{25}→Pro)	Leading sequence	81 recipients, 83 controls	No	25
TNF-α	-308 A/G	Promoter	147 recipients, 134 donors	No	42
TNF-α	-308 A/G	Promoter	81 recipients, 83 controls	No	25
IL-10	-1082 G/A	Promoter	81 recipients, 83 controls	Yes	25
IL-10	-819 C/T	Promoter	81 recipients, 83 controls	No	25
IL-1ß	+3953 C/T	Exon 5	81 recipients, 83 controls	Yes	25
IL-1ß	-31 C/T	Promoter	81 recipients, 83 controls	No	25
IL-1R1	+131 C/T	Promoter	81 recipients, 83 controls	No	25
IL-1RN	+8061 C/T	Intron 2	81 recipients, 83 controls	No	25
IL-1RN	86 bp VNTR	Intron 2	81 recipients, 83 controls	Yes	25
IL-2	-330 T/G	Promoter	301 recipients	No	40
IFN-γ	CA repeat	First intron	301 recipients	No	40
ICAM-1	E469/K469 (C/T)	Exon 6	82 recipients, 96 donors, 101 controls	Yes	18
ICAM-1	G241/R241 (C/T)	Exon 4	82 recipients, 96 donors, 101 controls	Yes	18
E-selectin	L554/F554	EGF domain	82 recipients, 96 donors, 101 controls	No	18
E-selectin	S128/R128	Exon 4/EGFdomain	82 recipients, 96 donors, 101 controls	No	18
L-selectin	F206/L206	Exon 6/EGF domain	82 recipients, 96 donors, 101 controls	No	18
L-selectin	P213/S213	Exon 6/EGF domain	82 recipients, 96 donors, 101 controls	No	18
PECAM-1	80Val-125Val/80Val-125Leu/80Met-125Val/80Met/125Leu	Exon 3	82 recipients, 96 donors, 101 controls	No	18
PECAM-1	Arg670/Gly670	Exon 12	82 recipients, 96 donors, 101 controls	No	18
PECAM-1	Asp563/Ser563	Exon 8	82 recipients, 96 donors, 101 controls	No	18
IL-6	-174 G/C	Promoter	81 recipients, 83 controls	No	25
HO-1	GT repeat	Promoter	292 recipients, 243 donors	No	44, 45

For associations, see text for details.

whether the Leu or the Pro allele is responsible for higher production.[21, 26] From the results of the above studies on SNP's in the TGF-ß1 gene, no uniform conclusion can be drawn.

For the ICAM-1 SNP's in exon 4 (G241R, Glycine 241Arginine) and exon 6 (E469K, Glutamic acid 469 Lysine), the genotypes of the donor were associated with freedom from TCAD. As mentioned before, it is currently unknown whether these SNP's are functional. There was a predominance of Glu469 in non-TCAD vs TCAD donors and controls. In addition, a decreased frequency of the combination of Gln241 and Lys469 was observed in non-TCAD donors compared with the TCAD donor group.[18]

For the IL-1RN repeat, four alleles were observed (allele 1, 4 bp; allele 2, 2 bp; allele 3, 5 bp and allele 4, 3 bp) and for analysis, allele 2, 3 and 4 were combined. Allele 1 was more frequently seen in patients with TCAD, especially in the homozygotic state. When this repeat was analyzed in conjunction with the IL-10 -1082 G/A SNP, allele 2 (combined with alleles 3 and 4) was associated with freedom from TCAD, preferentially in IL-10 A-allele carriers. When this repeat was analyzed in a subgroup of patients who had suffered multiple acute rejection episodes, the combination of homozygosity IL-1B +3953 C with homozygosity for the IL-1RN allele 1 was found to be a significant risk factor for TCAD.[25] It seems that both the 86 bp repeat in the IL-1RN and the IL-1B +3953 SNP are functional. Both genes cooperate in regulating the production of IL-1RN and IL-1ß expression.[27, 28] In the physiological context, the interaction between the IL-1, IL-1R and IL10 also remains to be investigated.

Renal failure

Renal insufficiency after heart transplantation is a frequently seen complication caused by the use of immunosuppressive drugs, mainly of calcineurin inhibitors. This renal failure is characterized by interstitial fibrosis, tubular atrophy, glomerular vascular lesions and accumulation of extracellular matrix. [29] It has been shown that cyclosporin A increases TGF-ß expression while a correlation exists between TGF-ß levels and renal failure.[30, 31] Only two polymorphisms (Leu^{10}→Pro and Arg^{25}→Pro) in relation to renal failure after heart transplantation are described. Although both were found to be associated with renal insufficiency, these findings were not consistent (Table 4).

Lácha et al. found that the decline in renal function was more evident in patients carrying the Leu allele at codon 10 compared to the Pro allele. They also associated homozygosity for the Arg allele in codon 25 with a poor renal function. This was even more pronounced in patients carrying both alleles. The worst

prognosis of renal function was found in patients homozygous for Leu10 and homozygous for Arg25.[6] However, this finding is not universal while also an opposite relation was found for the Leu^{10}→Pro SNP. Carriers of the Pro allele at this position had more often renal failure compared to carriers of the Leu allele.[32] Like in TCAD, it is difficult to draw conclusions from the results of these studies on TGF-ß1 polymorphisms.

Table 4. Cytokine gene polymorphisms in heart transplant recipients and their donors in relation to renal failure

Gene	Polymorphism	Location	Number of recipients, donors or controls	Association	Ref
TGF-ß1	+869 T/C (Leu^{10}→Pro)	Leading sequence	175 recipients, 268 controls	Yes	6
TGF-ß1	+869 T/C (Leu^{10}→Pro)	Leading sequence	168 recipients, 94 controls	Yes	32
TGF-ß1	+915 G/C (Arg^{25}→Pro)	Leading sequence	175 recipients, 268 controls	Yes	6
TGF-ß1	+915 G/C (Arg^{25}→Pro)	Leading sequence	168 recipients, 94 controls	No	32
TGF-ß1	+915 G/C (Arg^{25}→Pro)	Leading sequence	121 recipients	No	43

For associations, see text for details.

Discussion

While summarizing and comparing the data from the literature concerning cytokine gene polymorphisms in relation to cause of heart failure leading to transplantation and complications after heart transplantation, we encountered a number of problems.

A clinically important issue making it difficult to compare studies is that definitions for complications are not consistent. A typical example comes from the studies concerning acute rejection after heart transplantation. Some groups define acute rejection as ISHLT grading ≥2 while others as ≥3A. Non-rejectors were defined as no AR, while others included also patients with one or even two acute rejection episodes in this group. Also the time scale in which AR episodes was observed was inconsistent. In some studies acute rejections in the first year were included, while others included only acute rejections within the first three months or even the first four weeks after transplantation. Thus, although the grading of acute

rejection is standardized [8], the definition of rejectors and non-rejectors is arbitrarily chosen by the investigators. Similar problems arise with the other complications after heart and lung transplantation, e.g. decline of renal function or the diagnosis of renal failure.

Differences in immunosuppressive regimens, i.e., use and type of induction therapy, the scheme of maintenance therapy and dosage of immunosuppressants, might also have led to discrepancies between findings of different groups. The used immunosuppressive therapy might overrule the possible effect of the polymorphisms. For example, cyclosporin increases the TGF-ß1 production.[30], thus the usage or dosage of cyclosporin might be more important than polymorphisms in the TGF-ß1 gene. Another example of this are glucocorticoids that inhibit activation of NFκB, which is a transcription factor for many immunoregulatory genes.[33] The use of a regimen without glucocorticoids or the dosage used might play an important role in production of certain cytokines and subsequently in the development of complications and thus might overrule the effect of polymorphisms.

Another important observation is that many studies on polymorphisms have been reported by a single group only. A limited number of association studies were reported by more than one group, while very few of the associations or non-associations were confirmed by others. This can have several reasons.[34] First, there probably is a publication bias, as it is more difficult to publish negative results. Studies in which no association was found might even not be submitted for publication. Second, the ethnic composition of the study population might have influenced the results (bias by ethnicity). In the majority of studies, the composition of the study population was not described. It has been shown that ethnicity can strongly influence the distribution of gene polymorphisms, including those in cytokines.[35] So, it might be well possible that the discrepancies between studies might be due to differences in ethnical background of the studied individuals. Third, other generally not mentioned characteristics that influence the end point, like gender, recipient or donor age, HLA mismatches, cold ischemia time, CMV disease might subsequently have influenced the outcome of the association studies. And fourth, the sizes of most study populations are very small and statistical power has generally speaking not been adequate. This might lead to over- or under-interpretation of results.

The problems in comparing results are once more underlined, when we attempted to describe results of different studies in a uniform way, so that we could accumulate the results and see whether we could observe a trend for a risk factor. Therefore, we calculated the allele frequencies in cases and control groups from

Table 5. Calculated recipient allele frequencies of polymorphisms in the TNF-α and IL-10 genes in relation to acute rejection

Reference	Allele frequencies		Analyzed groups	Definition of AR	Characteristics of studied population (i.e., ethnic origin, gender, age, HLA mismatches, cold ischemia time, etc.)	Medication
	TNF-α -308					
	A/A + A/G	G/G				
15	13 (32%)	32 (43%)	AR 0, 1, 2	AR: ISHLT≥2 in the first 3 months	Not described	Cyclosporine Azathioprine Prednisolone
	20 (49%)	31 (42%)	AR 3, 4			
	8 (19%)	11 (15%)	AR >4			
14	9 (38%)	42 (61%)	AR 0, 1	AR: ISHLT≥3A in the first year	Children, age not described	Majority Tacrolimus
	15 (62%)	27 (39%)	AR>1			
19	5 (38%)	16 (48%)	NSR	AR in the first 4 weeks NSR: ISHLT 0, 1A, 1B SR: ISHLT 2, 3A, 3B, 4	Not described	Cyclosporine Azathioprine Prednisolone (Including doses)
	8 (62%)	17 (52%)	SR			
13	37 (82%)	74 (100%)	Survived AR	AR: ISHLT≥3A. Patients who died from AR and who survived more than 6 months after AR	Not described	Cyclosporine Azathioprine Prednisolone rATG induction (Including doses)
	8 (18%)	0 (0%)	Died due to AR			
	IL-10 -1082					
	A/A	A/G + G/G				
15	8 (38%)	37 (49%)	AR 0, 1, 2	AR: ISHLT≥2 in the first 3 months	Not described	Cyclosporine Azathioprine Prednisolone
	13 (62%)	38 (51%)	AR 3, 4			
			AR >4			
14	9 (18%%)	17 (41%	AR 0, 1	AR: ISHLT≥3A in the first year	Children, age not described	Majority Tacrolimus
	42 (82%)	24 (59%))	AR>1			
39	9 (56%)	24 (44%)	AR: 0	AR: ISHLT≥3A in the first year	Not described	Not described
	7 (44%)	30 (56%)	AR: >0			

AR: acute rejection. ISHLT: International society for heart and lung transplantation. NSR: non significant rejection. SR: significant rejection.

the data for SNP's that are studied more then once. In Table 5, we show the results for two SNP's, IL-10 -1082 A/G and TNF-α -308 A/G. As the table shows, the above mentioned problems, i.e., different definitions of end-points, not described composition of the study population, different immunosuppressive regimens, small study groups, are clearly illustrated. Nonetheless, the 4 studies on the TNF-α -308 A/G promoter polymorphism all seem to indicate that the A-allele is associated with some kind of susceptibility for acute rejection. For the IL-10 -1082 A/G SNP, two out of three seem to indicate that the A-allele is a risk factor for development of acute rejection. However, with the information given in these articles, it is difficult to draw conclusions about consistency of associations with genetic risk factors for development of transplant related complications.

Complications occurring after receiving a transplant have a complex multigene basis. There are many cytokines implicated in these complications which act in a network with other cytokines and growth factors. In addition, many cytokines appear to be pleiotropic and overlap in function (redundancy). If a polymorphism is functional, this redundancy complicates interpretation of the functionality. Moreover, data about functionality of polymorphisms are most often based on *in vitro* data and these data are not consistent.[36, 37] The associations between the polymorphisms and *in vitro* production might not reflect what happens *in vivo*. Therefore, the definition of for example "high" or "low" producer alleles or genotypes is premature. So, in this review, we just described the studied alleles and genotypes. described by their localization within the gene, rather than by their presumed *in vitro* cytokine production profile.

This multigenic basis of transplant related complications also indicates that polymorphisms should be analyzed in combination. However, the study populations are too small to perform such analyses. Moreover, the interactions between different polymorphisms within on gene and the interactions between cytokines remain to be investigated, to establish the net effect and influence on development of complications after transplantation. Currently, other known risk factors for transplant related complications, such as donor age, cold ischemia time, HLA mismatches etc., should be taken into account.

We conclude from the results described in the literature and summarized in this review, that it is not yet possible to use cytokine and protective gene polymorphisms to find genetic risk factors for the cause of heart failure, i.e., dilated cardiomyopathy or ischemic heart disease, or to predict the outcome after heart transplantation and to give guidelines for monitoring and treatment. If we continue performing association studies in the way we do now, this will never be achieved. When we want to apply genetic information in the clinical practice,

larger studies must be performed. Polymorphisms in candidate genes should be studied by a number of groups, using identical definitions for complications, and as far as possible also comparable patient and donor characteristics. Furthermore, negative association studies should be published or reported. We propose to start a database in which polymorphism allele and genotype frequencies, standardized definitions for complications after transplantation and characteristics of the study population should be collected. In this way, we can perform meta-analyses, which might contribute in establishing true genetic risk factors for complications after heart and lung transplantation. Once this has been accomplished, this may guide us in the monitoring and treatment of our patients.

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Summary and conclusions

Chapter 11

Summary

Inflammation is a characteristic feature of heart failure and of complications after heart transplantation. These inflammatory responses are regulated by cytokines and stress proteins. The production and function of cytokines and stress proteins can be controlled by genetic variations, polymorphisms. In this thesis, we attempted to identify genetic risk factors for the underlying diseases of the heart transplant patients, ischemic heart disease and dilated cardiomyopathy and for complications after heart transplantation, acute rejection, graft vascular disease and renal failure. For that reason, we studied polymorphisms of relevant cytokines (i.e., IL-2, IFN-γ and TGF-ß1) and a stress protein gene (HO-1).

In **chapter 1**, a general introduction is given on the role of cytokines and stress proteins in the pathogenesis of heart failure and transplant related complications. Polymorphisms in the genes of these mediators can influence their production or function. In the first section of the thesis, we explained what polymorphisms are and described the most common polymorphism classes. Genetic polymorphisms might cause differences in susceptibility to cardiovascular disorders and complications after transplantation, and also in responsiveness to treatment and in the onset of transplant related complications.

In **chapter 2**, the objectives of the association studies are described.

In **chapter 3**, we studied whether we could find an association between a dinucleotide repeat polymorphism $(CA)_m(CT)_n$ in the 3'flanking region of the IL-2 gene and acute rejection after heart transplantation. We compared the allele and genotype frequencies of patients with and without acute rejection episodes and showed that one allele (allele 135) of the IL-2 repeat was more frequently observed in patients without acute rejection episodes. In addition, we found an interaction between this 135 allele and HLA-DR mismatching with the donor. Recipients without allele 135 rejected their grafts irrespective of the number of mismatches. In contrast, patients with allele 135 experienced less acute rejections when they had zero or one mismatch, compared to patients with two mismatches or patients without allele 135.

Chapter 4 describes the results of an association study of polymorphisms in the IL-2 and IFN-γ genes in relation to both acute rejection and graft vascular disease. The polymorphisms studied were a SNP at position -330 T/G in the promoter of the IL-2 gene and a $(CA)_n$ repeat in the first intron of the IFN-γ gene. For both polymorphisms, we compared allele and genotype distributions of patients with and without acute rejection episodes in the first post transplant year. We also analyzed the polymorphisms in relation to the number and the time to the first

acute rejection episode. Finally, we analyzed both polymorphisms in relation to graft vascular disease. We were not able to find associations between the IL-2 or IFN-γ gene polymorphisms and the studied complications after heart transplantation.

In **chapter 5, 6, and 7**, we investigated two polymorphisms (Leu^{10}→Pro and Arg^{25}→Pro) in the leader sequence of the TGF-ß1 gene and tried to find associations between these SNP's and heart failure, transplant atherosclerosis and renal failure after heart transplantation. In **chapter 5**, we described the results of the TGF-ß1 polymorphisms in heart failure. We compared the TGF-ß1 allele and genotype distribution of patients with ischemic heart disease (IHD) or with dilated cardiomyopathy (CMP). We found a significant association between the SNP that changes the amino acid at codon 10 from Leucine to Proline, and CMP. We observed an excess of heterozygotes in the patients transplanted for CMP compared to patients with ischemic heart disease and healthy controls. For the SNP in codon 25, we could not find an association with the indications for transplantation. In **chapter 6**, we investigated whether the SNP's in the TGF-ß1 gene are risk factors for the development of graft vascular disease. Therefore, we measured the polymorphisms in both recipient and donor DNA. In an univariate analysis a difference in genotype distribution between patients with and without GVD was found. More patients with a Pro/Pro genotype were observed in the group with GVD. To establish whether this genotype was indeed a risk factor for the development of GVD, we performed a multivariate analysis with other known risk factors included. Again, we found that the TGF-ß1 codon 10 polymorphism is a risk factor for GVD, whereby the patients with the Pro/Pro genotype were at greatest risk. For the codon 25 polymorphisms we did not find an association with GVD. The third study on TGF-ß1 SNP's is described in **chapter 7**. In this chapter we investigated both polymorphisms in the TGF-ß1 gene in relation to renal failure after heart transplantation. We compared genotype distributions of patients with and without renal failure at 1 year, 3 years, 5 years and 7 years after transplantation. Again, an association with the polymorphism at codon 10 was found. At 7 years post transplantation, more carriers of the Pro allele suffered from renal insufficiency than patients carrying two copies of the Leu allele. For the polymorphism at codon 25, we did not find an association.

In **chapter 8** we described a study on the role of HO-1 and the possible mechanism by which HO-1 acts in the initiation, development and progression of TCAD. We showed a higher intragraft mRNA expression of HO-1 at one week and 10 months after transplantation in patients with signs of TCAD on their first year angiogram compared to patients without. Also TGF-ß mRNA expression was higher in biopsies

from TCAD positive patients at both time points. The higher HO-1 expression level in the week one biopsies was accompanied by a pro-apoptotic shift in the balance between the anti- and pro-apoptotic markers Bcl-2 and Bax. This suggests that patients who are going to develop TCAD might be prone for apoptosis via the mitochondrial pathway in the early period after transplantation. The higher HO-1 expression in the TCAD positive patient group could also be the result of the genetic capacity to produce higher amounts of HO-1. However, we could not find an association between the functional, with differences in enzymatic activity associated, repeat polymorphism $(GT)_n$ in the HO-1 gene and the development of TCAD. In **chapter 9** we attempted to identify whether this repeat was associated with the primary diseases of heart transplant patients and with AR and TCAD after transplantation. No association between this $(GT)_n$ polymorphism and primary diseases of the transplant recipients, AR or TCAD after heart transplantation was observed.

In **chapter 10** contains an overview of the studies of cytokine and stress protein gene polymorphisms in heart transplantation published in the international literature. We attempted to compare the studies to identify true genetic risk factors for the primary diseases of heart transplant patients and transplant related complications and whether we could use these potential risk factors in the clinical practice.

Conclusion and perspectives

The association studies of cytokine and stress protein gene polymorphisms in relation to the cause of heart failure leading to transplantation and transplant related complications described in this thesis, resulted in several associations. We can consider these associations as potential genetic risk factors, but have to interpret these results with caution. To ascertain whether these associations are true risk factors, the polymorphisms should be studied by other groups and with the results we can then perform meta-analyses. The outcome of these analyses might enable us to draw stronger and more unambiguous conclusions. However, it seems unlikely that, on the basis of single polymorphisms, we will be able to design individual treatment strategies. In the pathogenesis of heart failure and the different complications after transplantation, cytokines act in a network and gene polymorphisms might not have the same impact on the different outcomes. Therefore, combinations of associated polymorphisms should be made to create genetic risk profiles of both recipient and donor. These genetic profiles might then

enable us to identify transplant patients at risk for specific complications. These patients can then be monitored more intensively and treated earlier to prevent or avoid serious consequences of this complication. It might also be helpful in selecting individuals in who lower dosage of immune suppression can be used or for drug withdrawal protocols.

Hoofdstuk 12

Samenvatting

Samenvatting

Harttransplantatie is de laatste behandelingsmogelijkheid voor patiënten met eindstadium hartfalen. De meeste patiënten die getransplanteerd worden lijden aan hartfalen dat veroorzaakt wordt door gedilateerde cardiomyopathie, ischemisch hartfalen, kleplijden of door een aangeboren hartafwijking. Het succes na harttransplantatie is de laatste jaren sterk verbeterd door de ontwikkeling van effectievere en specifiekere afweeronderdrukkende geneesmiddelen. Helaas treden er zowel op korte als op lange termijn na transplantatie nog steeds complicaties op, zoals acute afstoting, infecties, versnelde coronair sclerose, nierfalen en maligniteiten.

Na harttransplantatie kan acute afstoting plaatsvinden in de eerste weken tot maanden na transplantatie. Acute afstoting wordt behandeld met extra afweeronderdrukkende medicijnen. Helaas neemt door het gebruik van deze afweerremmende geneesmiddelen het risico van het ontstaan van infecties en kanker toe. Verder is gebleken dat acute afstoting een risico factor is voor het ontstaan van versnelde coronair sclerose. Om deze complicaties te voorkomen is het beter om acute afstoting te voorkomen.

Versnelde coronair sclerose wordt gekenmerkt door vernauwing van de kransslagaders en van de kleine bloedvaatjes in het donorhart. Dit proces ontwikkelt zich in maanden tot jaren na transplantatie. Uiteindelijk vertonen bijna alle donorharten tekenen van deze complicatie. Versnelde coronair sclerose beïnvloedt de lange termijn overleving na harttransplantatie, ongeveer 30% van de ontvangers van een donorhart overlijdt aan deze complicatie. Het proces van versnelde coronair sclerose is onomkeerbaar en tot op heden niet te behandelen.

Bij hartfalen en bij complicaties die optreden na harttransplantatie zijn immunologische processen en ontstekingsreacties betrokken. Cytokinen en stress eiwitten spelen hierbij een belangrijke rol. Cytokinen zijn eiwitten die interacties tussen cellen reguleren. Door binding aan een receptor op het celoppervlak geven ze signalen door naar de kern van de cel, waardoor weer andere cytokinen geproduceerd worden. Vervolgens worden cellen geactiveerd en aangezet tot delen en differentiatie of gaan juist dood. Stress eiwitten zijn eiwitten die bijvoorbeeld door een tekort of juist een teveel aan zuurstof geactiveerd worden en zorgen dat de schade die dit tekort of teveel aan zuurstof veroorzaakt beperkt blijft.

In de genen van cytokinen en stress eiwitten bevinden zich polymorfismen. Polymorfismen zijn variaties in de DNA sequentie, die in minstens 1% van de populatie voorkomen. Elke variant van een polymorfisme wordt een allel genoemd.

Voor ieder polymorfisme heeft een individu 2 allelen, elk afkomstig van één ouder. De combinatie van 2 allelen wordt een genotype genoemd. Er zijn verschillende soorten polymorfismen. De meest voorkomende zijn de "single nucleotide polymorphisms" of "SNP", deze verschillen 1 basenpaar in het genomisch DNA, bijvoorbeeld ATGCCT→ATGGCT. De tweede soort zijn de "simple repeat polymorphisms", dit zijn herhalingen van meerdere basenparen waarbij de herhalingen verschillen in aantal, bijvoorbeeld CACA of CACACACA.

Een derde categorie zijn de "insertions/deletions". Bij deze groep is een basenpaar of een aantal basenparen ingevoegd of weggevallen. Een voorbeeld hiervan is AATGCA→ A.TGCA. Polymorfismen kunnen functioneel of anoniem zijn. Een functioneel polymorfisme heeft biologische consequenties. Ze kunnen o.a. zorgen voor verschillen in productie van cytokinen of stress eiwitten, kunnen de structuur, vouwing of lading van een eiwit veranderen en daarmee de functie. Anonieme polymorfismen hebben geen biologische gevolgen, maar kunnen gekoppeld zijn aan andere, wel functionele, polymorfismen.

Polymorfismen kunnen een risicofactor vormen voor het ontstaan van hartfalen, hetzij veroorzaakt door ischemie of door gedilateerde cardiomyopathie. Tevens kunnen polymorfismen betrokken zijn bij het ontstaan van complicaties na harttransplantatie.

Het doel van het onderzoek beschreven in dit proefschrift is het vinden van genetische risicofactoren voor het ontstaan van de ziekte leidend tot hartfalen (ischemisch hartfalen of gedilateerde cardiomyopathie) en complicaties na harttransplantatie (acute afstoting, versnelde coronair sclerose en nierfalen). We hebben daartoe associatiestudies uitgevoerd met polymorfismen in de genen van de cytokinen, Interleukine (IL)-2, Interferon (IFN)-γ en Transforming growth factor (TGF)-ß1 en het stress eiwit Haem Oxygenase (HO)-1.

In **hoofdstuk 3** is de relatie tussen een repeat polymorfisme $(CA)_m(CT)_n$ in de 3'flanking region van het IL-2 gen en acute afstoting na harttransplantatie beschreven. We hebben het voorkomen van allelen van dit polymorfisme vergeleken tussen patiënten met en zonder acute afstoting in het eerste jaar na harttransplantatie. We vonden dat één van de allelen (allel 135) vaker voorkwam bij ontvangers van een donorhart die geen afstotingsperiode doormaakten. Verder vonden we een interactie tussen dit allel en het aantal HLA-DR mismatches met de donor. Het bleek dat patiënten met het IL-2 allel 135 en weinig HLA-DR (0 of 1) mismatches minder vaak afstotingsperioden doormaakten vergeleken met patiënten met veel mismatches (2) of patiënten zonder allel 135. Dit suggereert dat matchen voor HLA-DR om het aantal acute afstotingen te verminderen, alleen

effectief zou zijn, als allel 135 van de $(CA)_m(CT)_n$ repeat in het IL-2 gen aanwezig is.

In **hoofdstuk 4** zijn de resultaten beschreven van 2 polymorfismen die we onderzocht hebben in relatie tot acute afstoting (in het eerste jaar) en versnelde coronair sclerose (diagnose gesteld op een jaar na transplantatie, mbv een coronair angiogram). Het eerste polymorfisme was een SNP (positie -330 T/G) in de promoter van het IL-2 gen en het tweede een repeat $(CA)_n$ in het eerste intron van het IFN-γ gen. Beide polymorfismen zijn geassocieerd met verschillen in productie van de betreffende cytokinen. We hebben bij patiënten met of zonder acute afstoting gekeken naar de relatie van de polymorfismen tot het aantal afstotingen en tot de tijd tot de eerste afstoting onderzocht. Voor versnelde coronair sclerose vergeleken we patiënten met en zonder deze complicatie. Voor beide polymorfismen werden geen associaties gevonden met de bestudeerde eindpunten.

In de volgende 3 hoofdstukken hebben we 2 SNP's in het signaal peptide van het TGF-ß1 gen bestudeerd. Deze 2 SNP's (+869 T/C, Leu^{10}→Pro en +915 G/C, Arg^{25}→Pro) veranderen aminozuren in het signaalpeptide van het TGF-ß1 gen en zijn geassocieerd met veranderde uitscheiding van het TGF-ß1 eiwit. In **hoofdstuk 5** hebben we deze polymorfismen onderzocht in relatie tot de oorzaak van hartfalen leidend tot transplantatie. We hebben allel en genotype frequenties van deze SNP's vergeleken tussen patiënten met ischemisch hartfalen en gedilateerde cardiomyopathie. We vonden een associatie tussen het Leu10Pro polymorfisme en gedilateerde cardiomyopathie. In deze patiëntengroep zaten meer mensen met een heterozygoot genotype vergeleken met de ischemisch hartfalen groep en gezonde controles. Dit suggereert dat TGF-ß1 een rol speelt in de pathogenese van gedilateerde cardiomyopathie en dat het codon 10 polymorfisme een risicofactor is voor het ontstaan van deze ziekte. In **hoofdstuk 6**, hebben we de relatie van deze SNP's met versnelde coronair sclerose (diagnose op één jaar) bestudeerd. Opnieuw hebben we patiënten met of zonder versnelde coronair sclerose de allel en genotypenverdeling met elkaar vergeleken. We vonden opnieuw een associatie met het Leu^{10}→Pro polymorfisme. In de groep met versnelde coronair sclerose bevonden zich meer mensen met het Pro/Pro genotype vergeleken met de mensen zonder de complicatie. Ook als we het polymorfisme bestudeerden in een multivariaat analyse, waarin ook andere risicofactoren voor deze complicatie geanalyseerd werden, bleek dat het Pro/Pro genotype een risicofactor voor deze complicatie was. En tot slot hebben we in hoofdstuk 7 de 2 polymorfismen in het TGF-ß1 gen bestudeerd in relatie tot nierfalen na harttransplantatie. Daartoe is het aanwezig zijn van de verschillende allelen en genotypen vergeleken in patiënten met of zonder nierfalen op 1, 3, 5 en 7 jaar. Het Leu10Pro polymorfisme

bleek geassocieerd te zijn met nierfalen na 7 jaar. Patiënten die drager zijn van het Pro allel hadden vaker nierfalen in vergelijking tot patiënten homozygoot voor het Leu allel.

Hoofdstuk 8 beschrijft de studie waarin gekeken is naar de mogelijke rol van het stress eiwit HO-1 bij het ontstaan van versnelde coronair sclerose. Een hogere mRNA expressie van HO-1 werd aangetoond in biopten van patiënten met versnelde coronair sclerose op één week en 10 maanden na harttransplantatie. Ook de TGF-ß mRNA expressie was hoger in deze biopten. De hogere expressie ging in de week 1 biopten gepaard met een verschuiving in de balans tussen anti- en pro-apoptotische markers Bcl-2 en Bax. Deze verschuiving vond plaats in de pro-apoptotische richting, wat duidt op een grotere gevoeligheid voor apoptose in de eerste periode na transplantatie. Er is dus meer schade door apoptose in de groep met versnelde coronair sclerose en HO-1 zou opgereguleerd kunnen zijn om verdere schade te voorkomen. Een andere verklaring voor de hogere HO-1 expressie is een verschil in aanleg om HO-1 te maken. We hebben daarom ook een repeat polymorfisme $(GT)_n$ in de promoter van het HO-1 gen bestudeerd. Dit polymorfisme is geassocieerd met verschillen in enzymatische activiteit. We konden echter geen associaties aantonen tussen dit polymorfisme en versnelde coronair sclerose. In **hoofdstuk 9** is dit polymorfisme nogmaals bestudeerd. Dit keer in relatie tot de oorzaak van hartfalen, acute afstoting en versnelde coronair sclerose. We hebben in deze studie geen associatie kunnen vinden tussen het polymorfisme en de reden voor transplantatie of complicaties na transplantatie. Het HO-1 $(GT)_n$ repeat polymorfisme lijkt dus geen risicofactor te zijn voor het ontstaan van hartfalen of complicaties na harttransplantatie.

Tot slot wordt in **hoofdstuk 10** geprobeerd een zo compleet mogelijk overzicht te geven van studies over cytokine genpolymorfismen en harttransplantatie, die tot nu toe in de internationale literatuur zijn verschenen. We wilden hiermee proberen vast te stellen of de tot nu toe gepubliceerde associatiestudies daadwerkelijk geleid hebben tot de identificatie van genetische risicofactoren en of deze potentiële risicofactoren bruikbaar zijn in de klinische praktijk. Het vergelijken van de studies was echter erg lastig. We kwamen een aantal problemen tegen: verschillen in definities van de complicaties, de grootte van de studiegroepen, het ontbreken van gegevens met betrekking tot de samenstelling van de studiegroepen, verschillen in afweeronderdrukkende medicijnen, het kleine aantal bevestigende studies door andere groepen en de waarschijnlijke publicatie bias.

In conclusie kunnen we zeggen dat de associatiestudies die beschreven zijn in dit proefschrift geleid hebben tot de identificatie van een aantal potentiële

risicofactoren voor het ontstaan van hartfalen en complicaties na harttransplantatie. We moeten deze resultaten echter wel met enige voorzichtigheid interpreteren. Om te bepalen of de associaties die we gevonden hebben echte risicofactoren zijn, waarvan gebruik gemaakt kan worden in de klinische praktijk, zullen andere groepen dezelfde polymorfismen moeten bestuderen. We zouden dan met de resultaten van alle studies een meta-analyse kunnen uitvoeren om daarmee in grotere groepen de gevonden associaties te kunnen bevestigen om eenduidige conclusies kunnen trekken. Het lijkt echter onwaarschijnlijk dat we op basis van de associaties met een enkel polymorfisme individuele medicijn protocollen kunnen maken. Bij de ontwikkeling van hartfalen en complicaties na harttransplantatie zijn namelijk meerdere genen en polymorfismen belangrijk, welke niet allemaal evenveel bijdragen aan elke ziekte of complicatie. Bijvoorbeeld, een polymorfisme dat voor een bepaalde complicatie gunstig zou kunnen zijn, zou voor een andere aandoening negatieve gevolgen kunnen hebben. Daarom zouden de associaties met de enkele polymorfismen gecombineerd moeten worden, zodat we een genetisch risico profiel kunnen maken. Dit genetisch profiel zou gebruikt kunnen worden om patiënten met een verhoogd risico voor een bepaalde complicatie te kunnen identificeren. Deze patiënten zouden intensiever gevolgd kunnen worden, waardoor er eerder met een mogelijke behandeling begonnen kan worden. We zouden dit profiel ook kunnen gebruiken om patiënten te identificeren die minder immuunsuppressie nodig hebben of voor protocollen waarin bepaalde medicijnen gestopt worden.

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About the author

Cécile Holweg was born in Lichtenvoorde, The Netherlands, on January 8, 1970. From 1982-1988 she attended secondary school (HAVO) at the "RK Scholengemeenschap Marianum" in Groenlo. Next, she started Higher Laboratory School (Hoger Laboratorium Onderwijs) at the "Rijkshogeschool IJselland" in Deventer, which she completed in 1992. The practical training for this education took place at the department of Pathology of the National Institute of Health and Environmental Protection (RIVM) in Bilthoven. She worked on a project which aimed to develop a vaccine against cervical cancer. After graduation, she started working as a technician at the department of Virology (now the Laboratory for Infectious Diseases) of the same institution. First, she participated in a clinical trial studying the efficacy of an HIV medication regimen. This project was in collaboration with the University Medical Center in Utrecht. Subsequently, she worked on a project for the worldwide eradication of the Poliovirus. From 1996 untill now, she has been working at the Transplantation Laboratory of the department of Internal Medicine of the Erasmus MC (University Medical Center) in Rotterdam. There, she contributed to different projects for which she performed various molecular biological tests. In 1998 she started studying cytokine and stress protein gene polymorphisms in relation to heart failure and complications after heart transplantation. This study was performed under supervision of Dr. Carla Baan and Prof. Dr. Willem Weimar and the results are described in this thesis.